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Osborne, T. B. & Mendel, L. B. [1914*a*]. *J. biol. Chem.* **17**, 325.
— — [1914*b*]. *J. biol. Chem.* **18**, 1.
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The Structure of Arachidonic and Linoleic Acids

By C. L. ARCUS and I. SMEDLEY-MACLEAN, *The Lister Institute, Chelsea Bridge Road, London, S.W. 1*

(*Received 3 September 1942*)

Early in 1940 Shinowara & Brown described the results of the ozonolysis of methyl arachidonate and of its oxidation by $KMnO_4$ in acetone solution. As a result of their experiments they formulated arachidonic acid as $\Delta^{6.10.14.18}$-eicosatetrenoic acid (I):

$CH_3.CH{:}CH.CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH{:}CH.$
$CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CO_2H$ (I)

They emphasized that this structure was not fully confirmed and must be regarded only as a tentative suggestion. Before the appearance of this paper, Dolby, Nunn & Smedley-MacLean had for some time been engaged in elucidating the structure of this acid, using the method of alkaline permanganate oxidation and as their results were entirely at variance with those obtained by Shinowara & Brown, they published a preliminary account [1940] indicating that arachidonic acid was $\Delta^{5.8.11.14}$-eicosatetrenoic acid (II):

$CH_3.CH_2.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.$
$CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CO_2H$ (II)

The evidence for this structure was that glutaric and succinic acids were the only dibasic acids isolated; the amount of glutaric acid obtained was very small but it gave no depression in melting-point when mixed with the pure acid. From the other end of the molecule a fraction of volatile fatty acids was obtained which behaved like a mixture of valeric and caproic acids. The melting-point of the *p*-bromophenacyl caproate separated by fractional crystallization was not raised to that of the pure compound, and it was considered desirable at some future time to confirm this result. The authors felt justified, however, in proposing the formula (II) for arachidonic acid.

From the biological point of view, the structure of arachidonic acid is of considerable importance. According to the results of Smedley-MacLean & Hume [1941] the rat synthesizes clupanodonic acid (docosapentenoic acid) only if linseed oil acids are supplied, and it is considered probable that these acids are also the precursors of arachidonic acid in the body. If the formula of Dolby *et al.* were correct, the 11 terminal carbon atoms of arachidonic and linoleic acids would be identically linked and the biological synthesis would involve the addition of 2 carbon atoms at the carboxyl end of the molecule and the introduction of double bonds at the 5.6 and 8.9 positions of the C_{20} chain or in the 3.4 and 6.7 positions of the C_{18} chain. On the other hand, the tentative formula suggested by Shinowara & Brown bore no resemblance to that of linoleic acid and did not contain the structure

$$—CH{:}CH.CH_2.CH{:}CH—$$

characteristic of the linseed acids. A comparison of the ozonolysis products of methyl arachidonate and ethyl linoleate was therefore undertaken by the present authors, together with an acetone-permanganate oxidation of the former, and the investigation had been almost completed when a communication from Mowry, Brode & Brown [1942] appeared in which the formula (I) tentatively suggested by Shinowara & Brown was withdrawn and that proposed by Dolby *et al.* (II) entirely confirmed. The later work of the American authors had been carried out on a large scale, using 197 g. of arachidonate in place of 11 g. employed in our experiments, enabling them to isolate relatively large quantities of purified oxidation products.

An eicosatetrenoic acid has been isolated by Toyama & Tsuchiya [1935] from fish oil which gave on oxidation only acids containing 4 carbon atoms. They therefore considered it to be $\Delta^{4.8.12.16}$-eicosatetrenoic acid which contains 4 of the groupings

$$—CH{:}CH.CH_2.CH_2—$$

and in which the characteristic linseed acid structure is absent. This acid has been designated arachidonic acid, but it seems certain that it is not identical with the arachidonic acid prepared from ox suprarenal fat, and it is unfortunate that the same name should be employed. We suggest that the name arachidonic acid be reserved for that originally isolated by Hartley [1909] from pig's liver, to which the constitution $\Delta^{5.8.11.14}$-eicosatetrenoic acid is assigned, and that the name should not be used to describe the acid obtained from fish oil.

A survey of the literature showed that apparently the only work on the ozonolysis of linoleic acid was by Takahashi [1921] whose results cannot be reconciled with the commonly accepted formula. This provided an additional reason for completing the present work. Takahashi ozonized the acid prepared from soya-bean and rice-bean oils; among the products he identified normal butyric acid and the corresponding aldehyde, glutaric acid and its dialdehyde, azelaic acid and its half aldehyde. He therefore represented the double bonds as occurring

in the 9:10 and 14:15 positions (III) instead of in the 9:10 and 12:13 positions (IV) as has been generally accepted:

$$CH_3CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (III)$$

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (IV)$$

Takahashi stated that the tetrabromo- and tetrahydroxy-stearic acids obtained from his linoleic acid were identical with those prepared from the linoleic acid of poppyseed oil. Haworth [1929] prepared linoleic acid from both soya bean and poppyseed oils, and obtained products by oxidation with acetone-permanganate, and with alkaline permanganate, which were in agreement with formula (IV). Birosel [1937] found the linoleic acid prepared from soya bean oil identical with that from cottonseed oil; both gave the normal tetrabromo- and tetrahydroxy-stearic acids. Ethyl linoleate has now been prepared from linoleic acid tetrabromide (M.P. 114–115°), obtained from maize oil, and on ozonolysis it gave products in agreement with formula (IV) ($\Delta^{9.12}$-octadecadienoic acid). We therefore do not confirm the results of Takahashi.

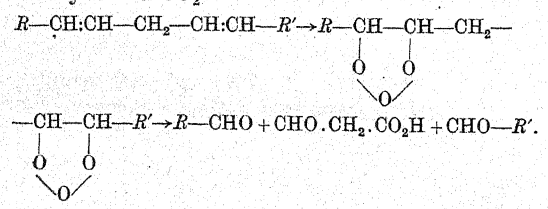
Erdmann, Bedford & Raspe [1909] have ozonized linolenic acid (V) and its esters:

$$CH_3CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (V)$$

They adopted the view of Harries that the ozonide decomposes giving an atom of nascent oxygen for each ozonide group, and that this oxygen may react with H_2O forming H_2O_2 or may oxidize the aldehydes to acids; under these conditions the lower aliphatic aldehydes are those most easily oxidized. Further oxygen is available because linolenic acid and its ethyl ester, when ozonized in chloroform solution, yield the ozonide peroxide; the increase in weight noted indicates this to be the case also for ethyl linoleate. The ozonide of methyl arachidonate was decomposed without removal of solvent but it is likely that it also is an ozonide-peroxide. Erdmann *et al.* [1909] found that the grouping

$$—CH{:}CH.CH_2.CH{:}CH—$$

yielded an ozonide which, with water, gave formylacetic acid, the latter decomposed at 60° to acetaldehyde and CO_2:



In our experiments, on decomposing the ozonides, acetaldehyde was trapped in 2:4-dinitrophenylhydrazine solution and CO_2 in NaOH solution.

Methyl arachidonate was prepared from arachidonic acid octabromide obtained from ox suprarenal fat kindly supplied to us by Messrs Allen and Hanburys Ltd. It had the characteristics recorded in the experimental portion. The observed molecular refractivity was 100·4 units, the calculated value being 99·05. It is considered from this result that in pure methyl arachidonate the double bonds are not conjugated, and that the slight exaltation shown is to be ascribed to the incidence of rearrangement of double bonds into conjugated positions in a fraction of the ester during distillation; this is regarded by Farmer & van den Heuvel [1938] as unavoidable except in molecular stills. The exaltation may be due in part to anomalous dispersion, since the refractive index for λ_{5461} was found to be lower than that for λ_{5893}. The products isolated were identified by the melting-point and by the mixed melting-point with an authentic specimen, the dibasic acids directly, the monobasic acids after conversion into *p*-bromophenacyl esters, and the aldehydes after conversion into 2:4-dinitrophenylhydrazones. The amounts of the products were estimated as far as possible, and where the yields are considerable, though none are nearly quantitative, they are recorded in Table 1; in all three experiments the only volatile acids found were caproic and acetic, and on the assumption that only these were present the quantities given in Table 1 were calculated from the weights and equivalent weights of the various sodium salts.

Table 1. *Ozonolysis of methyl arachidonate*

	Methyl arachidonate	CO_2	Acetaldehyde 2·4-dinitrophenylhydrazone	Na caproate	Na acetate
Wt. (g.)	5·74	0·966	1·976	0·686	2·046
Moles ($\times 10^{-3}$)	18·0	22·0	8·8	5·0	25·0
Ratio	1·0	1·2	0·5	0·3	1·4

Glutaric acid was identified and the presence of caproic aldehyde indicated. The figure given for sodium acetate must be regarded as approximate.

Oxidation of methyl arachidonate by acetone-$KMnO_4$

	Methyl arachidonate 5·0 g. less 1·0 g. recovered	CO_2	Acetic acid	Caproic acid
Wt. (g.)	4·0	2·38	0·86	0·82
Moles ($\times 10^{-3}$)	12·6	54·1	14·3	7·1
Ratio	1·0	4·3	1·1	0·6

Oxalic acid was identified and succinic acid and caproic acid were indicated. A hydrocarbon identified as n-$C_{27}H_{56}$, and polymerized material (0·63 g.) were also isolated.

Ozonolysis of ethyl linoleate

	Ethyl linoleate	CO_2	Na caproate	Azelaic acid
Wt. (g.)	3·37	0·259	0·848	0·753
Moles ($\times 10^{-3}$)	10·9	5·9	6·1	4·0
Ratio	1·0	0·5	0·6	0·4

Acetaldehyde 2:4-dinitrophenylhydrazone was identified.

The caproic acid found in the ozonolysis of methyl arachidonate indicates that the molecule contains the fragment

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH:$$

The non-volatile fraction was easily oxidized by permanganate giving a product containing an ester group and a carboxyl group, and saponification of this material gave glutaric acid as the only product detected. This sequence of reactions is consistent with the conversion of (impure) glutaric half-aldehyde methyl ester into methyl hydrogen glutarate and then to glutaric acid indicating the terminal portion of the molecule to be

$$:CH.CH_2.CH_2.CH_2.COOMe$$

The remaining 9 carbon atoms yielded 1·9 moles of acetyl radical (as acid and aldehyde) which certainly indicated 3 rather than 2 moles. The yield of CO_2 is low, but comparison with the ethyl linoleate experiment points to the presence of 3 moles of CO_2. Having regard to the results of Erdmann *et al.* [1909] three

$$:CH.CH_2.CH:$$

groups are present and must be adjacent. From these results it is concluded that arachidonic acid has the formula (II).

Farmer & van den Heuvel [1938] oxidized with acetone-permanganate a methyl docosahexenoate containing several

$$:CH.CH_2.CH:$$

groups and found that the ethylenic carbons were oxidized to carboxyl so that these groups gave (presumably via malonic acid) acetic acid. Haworth [1929] oxidized linoleic acid with this reagent, and found a similar rupture of the ethylenic groups, but here the malonic acid, traces of which were detected, gave oxalic acid. We have oxidized methyl arachidonate under conditions resembling those of Farmer & van den Heuvel [1938]. The reaction did not proceed smoothly; 20 % of unoxidized ester was recovered after 37 hr., a considerable amount of polymerized material was formed, and a small quantity of a hydrocarbon, identified by melting-point and analysis as n-$C_{27}H_{56}$, was isolated. The last must have been formed under the somewhat strenuous and prolonged reaction conditions; it was not encountered during ozonolysis. It is considered that the methyl arachidonate was slowly oxidized at the double bonds, giving caproic acid, malonic acid (which mainly decarboxylated to give acetic acid but which was also oxidized to oxalic acid) and glutaric acid, which was further oxidized to succinic acid. (The reaction mixture becomes strongly alkaline and apparently hydrolyses the ester group.)

Ozonolysis of the ethyl linoleate yielded caproic acid, indicating the grouping

$$CH_3CH_2.CH_2.CH_2.CH_2.CH:$$

The sequence azelaic half-aldehyde ethyl ester, ethyl hydrogen azelate, azelaic acid could be followed fairly precisely giving the terminal part of the molecule as

$$:CH.(CH_2)_7.COOOEt$$

The acetaldehyde and CO_2 indicate the remaining 3 carbon atoms to have been

$$:CH.CH_2.CH:$$

The commonly accepted formula (IV) for linoleic acid is therefore confirmed.

EXPERIMENTAL

Melting-points and boiling-points are corrected.

Ozonolysis of methyl arachidonate. Methyl arachidonate was prepared by reduction of arachidonic acid octabromide (60 g.) with zinc (60 g.) in methyl alcohol (600 ml.) containing HCl and subsequent esterification according to Ault & Brown [1934]. It (6·25 g.) had b.p. 177–178°/0·35 mm., i.v. Hübl = 310 (calculated for $C_{19}H_{31}COOCH_3$, 319) and $n_D^{24°}$ 1·4828, $n_{5461}^{24°}$ 1·4811, $\delta_{4°}^{20°}$ 0·905, whence R_D obs. = 100·4; R_D calc. 99·0 [Eisenlohr, 1911] or 99·05 [Swietoslowski, 1920].

The ester (5·86 g.) was dissolved in chloroform (40 ml.), cooled in ice and ozonized by passing a current of 8 % ozone through the solution; portions of the solution were withdrawn for test with Br in CCl_4, until (after 22 hr.) no unsaturation remained. Water (45 ml.) was added to the ozonide solution, which was cloudy and smelt aldehydic. The whole was slowly heated to 98° in a distilling flask connected by ground joints to a condenser and a receiver, and thence to a wash bottle containing 600 ml. of saturated solution of 2:4-dinitrophenylhydrazine in 2N HCl, and a long wash tube containing N NaOH (175 ml.) in a column 26 cm. high; CO_2-free air was aspirated through the apparatus. The hydrazine solution was then removed and replaced by fresh reagent and steam passed, aspiration being continued. In this way CO_2, acetaldehyde, non-volatile, and volatile fractions were separated.

The ice-cooled NaOH solution was titrated with N H_2SO_4 using phenolphthalein and then methyl orange, and 0·966 g. CO_2 were found. A slight excess of H_2SO_4 was added and 87 % of the solution was distilled off; the distillate contained volatile acid equivalent to only 2 % of the found CO_2.

The 2:4-dinitrophenylhydrazine solution yielded crude acetaldehyde 2:4-dinitrophenylhydrazone (1·875 g. m.p. of the two ppts. 142–144° and 149–159·5°) from which fractional crystallization gave the pure compound m.p. 169° mixed m.p. 168·5–169° with authenic acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 169°).

The solution of non-volatile compounds was filtered from a small quantity of oily polymer, an equal volume of saturated brine added, and the

solution repeatedly extracted with ether, and dried with Na_2SO_4, yielding 1·53 g. of material. This was suspended in hot Na_2CO_3 solution and treated with 193 ml. $KMnO_4$ ($N/10$) to oxidize aldehyde to carboxyl, on the assumption that 80 % would be glutaric half-aldehyde methyl ester. The solution was acidified (H_2SO_4), extracted repeatedly with ether, and the extract washed, dried (Na_2SO_4) and evaporated. The residue was first titrated with, and then quantitatively saponified with, alcoholic potash. (Found: COOMe, 14; COOH, 42 %. Calc. for $HOOC.CH_2.CH_2.CH_2.COOMe$: COOMe, 40; COOH, 31 %.) The saponified solution, after dilution with water and evaporation of the alcohol, was acidified (H_2SO_4), saturated with salt, and extracted with ether, first by shaking and then for 8 hr. in a continuous extractor. The former extract gave a brown solid which, after crystallization from chloroform-light petroleum, was dissolved in hot water, filtered and evaporated; it then had m.p. 61–65·5° but could not be identified. The second extract gave a white solid which was similarly treated, then repeatedly recrystallized from benzene, and which yielded a small quantity of glutaric acid, m.p. 92·5–93·5° and mixed m.p. 95·5° with authentic glutaric acid (m.p. 97·5–98°).

To the distillate, consisting of chloroform and aqueous layers, more chloroform was added and the whole titrated with N NaOH, with shaking, in a separating funnel. The chloroform solution was separated, dried (Na_2SO_4) and the solvent removed, the latter was mechanically shaken with an excess of a solution of 2:4-dinitrophenylhydrazine in 2N HCl, when it gave a derivative, which after repeated crystallization, had m.p. 152–152·5° (0·101 g.) and was probably impure acetaldehyde 2:4-dinitrophenylhydrazone. The neutral residue, after removal of chloroform, was distilled, giving a small distillate, b.p. 70–130°; this furnished a 2:4-dinitrophenylhydrazone which after recrystallization had m.p. 100–101·5°. The mixed m.p. with authentic caproic aldehyde 2:4-dinitrophenylhydrazone (m.p. 109°) was 103–104°.

The aqueous solution after evaporation and drying to constant weight in a vacuum desiccator, gave 2·732 g. of sodium salt having equivalent 91·3 (from the amount of N NaOH used). (The same procedure for estimating the equivalent of sodium salts was employed a number of times below.) This was dissolved in water (110 ml.), acidified (H_2SO_4) and distilled; the first 26 ml. and the next 75 ml. of distillate were collected. The last was neutralized and yielded a sodium salt (1·378 g., equiv. 83·7), giving a *p*-bromophenacyl derivative which melted for the most part at 81–85° (*p*-bromophenacyl acetate has m.p. 87–87·5°), but which contained a higher melting impurity. We made a number of attempts but were unable to separate the latter. A portion of the sodium salt was refluxed with dilute H_2SO_4 and the solution distilled; the distillate on neutralization gave a sodium salt having equiv. 83·2 (sodium acetate has equiv. 82), but again we were unable to prepare a pure *p*-bromophenacyl ester.

The first fraction of distillate contained a layer of oily acid which was pipetted off, and the solution again distilled; 6·3 ml. of this distillate were taken, combined with the oily acid and neutralized giving 0·403 g. of a sodium salt, equiv. 127. It was converted into its *p*-bromophenacyl derivative which, after three recrystallizations, had m.p. 70·5–71·5° and mixed m.p. 71–71·5° with authentic *p*-bromophenacyl caproate of m.p. 71·5–72°.

Oxidation of methyl arachidonate by acetone-$KMnO_4$. Methyl arachidonate (5·0 g.) was dissolved in dry acetone (300 ml.) and heated under reflux in an apparatus in which the returning acetone percolated through $KMnO_4$. In the course of 37 hr. 36·6 g. of the last were used without the appearance of a pink coloration; since only 31·4 g. would be necessary to convert each ethylenic link into two carboxyl groups, the oxidation was stopped. The solution was filtered from the manganese mud and the latter washed with acetone. The acetone was evaporated and the residue taken up in light petroleum and washed with NaOH solution and with water (added to bulk aqueous extract below). After drying (Na_2SO_4) and removal of solvent, unchanged ester (1·0 g.) remained.

The manganese mud was dried and leached out with 500 ml. boiling water. The latter after filtration, evaporation and desiccation yielded 11·9 g. of salts (A). The desiccated manganese mud now weighed 19·6 g. The CO_2 content of these two fractions (which contained volatile organic acids) was determined as follows. An aqueous solution of the material was heated to boiling under reflux in a current of CO_2-free air, the top of the condenser being connected to a wash bottle containing $N/10$ $Ba(OH)_2$. Dilute H_2SO_4 was run into the solution and the liberated CO_2 swept through. The baryta solution was filtered and titrated against $N/10$ HCl. The difference from a blank gave the CO_2 trapped, and a correction for imperfections in this method was applied from control experiments with known quantities of carbonate. The extracted, dry, manganese mud and the salts (A) contained 48·9 and 119·4 mg. CO_2/g. respectively.

The salts (A) (3·7 g.) were dissolved in water, acidified (H_2SO_4) and steam distilled.

Successive fractions of distillate (ml.)	10	10	10	150	180
Na salt: wt. (mg.)	123	74	55	321	87
equiv.	103·5	98	97	90	172

A repetition with 7·65 g. of salts (A) yielded three fractions of sodium salts: 0·685 g., equiv. 93; 0·211 g.,

equiv. 86; 0·265 g., equiv. 115. The first of these fractions was dissolved in a minimum of water, acidified (H_2SO_4), extracted repeatedly with ether, and the extract dried (Na_2SO_4) and evaporated. The residue was fractionated by distillation through a series of three bulbs which were successively heated in an air-bath. The fraction (20 mg.) taken at air-bath temperature, 195–225°, was converted via the acid chloride to the anilide which had m.p. 79·5–81·0°, mixed m.p. 80–81·5° with caproic anilide (m.p. 96°), and mixed m.p. 55–56·5° with *n*-valeric anilide (m.p. 61·5–62°). The fraction was thus probably caproic acid. The mid-fraction of sodium salts (0·308 g.) yielded a *p*-bromophenacyl derivative (0·721 g.) of m.p. 70°; five recrystallizations gave *p*-bromophenacyl acetate, m.p. 83–83·5° and mixed m.p. 86° with the authentic compound (m.p. 87–87·5°).

There remained in the steam distillation flask a tar and an aqueous solution; the former (insoluble in benzene and in ether) was dissolved in acetone, dried (Na_2SO_4), and after removal of solvent weighed 0·404 g. The aqueous solution was extracted with ether in a continuous extractor for 22 hr. and with fresh solvent for a further 6 hr. On removal of the ether the residue was dissolved in hot acetone (in which nearly all of it dissolved), filtered, and cooled, when crystals, 49 mg., m.p. 55°, were deposited. After two recrystallizations from benzene-acetone, it had m.p. 57–58°. (Found: C, 84·6; H, 15·0 %. *n*-$C_{27}H_{56}$ has m.p. 58·5–59·5°, C, 85·2; H, 14·9 %.) Addition of two volumes of light petroleum to the acetone filtrate yielded an oil which crystallized. The product after draining and washing with ether had m.p. 99–100° and was dissolved in water, filtered, evaporated and desiccated over H_2SO_4. The residue (45·5 mg.) had m.p. 160–161·5° (decomp.); a portion was converted into the ammonium salt which yielded pyrrole on ignition with zinc dust, indicating succinic acid. A further portion was titrated with NaOH, giving equiv. 51·3, and the solution was acidified (acetic acid), treated with $CaCl_2$ solution, and the precipitate titrated in acid against $KMnO_4$ at 60°, whence 33·2 mg. (anhydrous) oxalic acid were obtained.

The acetone-light petroleum solution on evaporation yielded a residue (C_6H_6), which after washing had m.p. 99–100°. It was dissolved in water, filtered and evaporated and yielded hydrated oxalic acid (41 mg., equiv. 63·2).

Ozonolysis of ethyl linoleate. Corn (maize) oil acids (177 g.; i.v. Hübl = 114) were brominated (40 ml. bromine) in light petroleum solution. The solid product was recrystallized from hot benzene yielding tetrabromostearic acid m.p. 114–115°. It (44 g.) was reduced with zinc dust according to Brown & Fraenkel [1938]. The linoleic acid was esterified with EtOH-HCl and gave ethyl linoleate (12·8 g.), b.p. 173·5°/0·5 mm.; i.v. Hübl = 161 (calc. 164·8); this (3·5 g.) was ozonized as before, saturation being reached after 9 hr. The chloroform was removed as far as possible in a water pump vacuum at 40°, yielding a colourless oil (4·90 g. Theory, assuming a *bis*-ozonide-peroxide, 4·75 g.).

Cold water (35 ml.) was added to the ozonide and decomposition was allowed to proceed in apparatus similar to that used for methyl arachidonate except that five small wash bottles were employed, the first containing a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl, the second the same reagent in alcoholic H_2SO_4 and the last three *N* NaOH (75 ml. each). The water-bath was raised to boiling point over a period of 1 hr. and a slow current of CO_2-free air continued overnight. The contents of the 2:4-dinitrophenylhydrazine wash bottles were then replaced by fresh reagent in 2*N* HCl solution, and steam passed in place of the air current, aspiration through the wash train being continued. Finally the distillate and the solution of non-volatile material in the flask were both saturated with salt and extracted with ether and the latter dried (Na_2SO_4).

The NaOH solution was cooled and titrated with *N* HCl, using phenolphthalein and methyl orange successively and 0·259 g. CO_2 was found.

Of the 2:4-dinitrophenylhydrazine solutions, the alcoholic one gave no appreciable yield, whilst the first and second aqueous ones gave respectively two products (0·151 and 0·035 g.), m.p. 107–109° and 87°, which on recrystallization had m.p. 145·5–147·5° and 137–143°. These were combined and after four recrystallizations had m.p. 160° and mixed m.p. 168–169° with authentic acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 168·5–169°).

Evaporation of the ethereal solution of non-volatile products gave a semi-solid mass which was washed with ether-light petroleum yielding crude azelaic acid (0·122 g.), m.p. 100·5–102·5°. The residue from evaporation of the washings was suspended in hot Na_2CO_3 solution and titrated with $KMnO_4$ until the reaction slowed down (required 63·9 ml. of $KMnO_4$, 0·06*N*). The solution was acidified (H_2SO_4), extracted with ether, the extract washed, dried (Na_2SO_4), and evaporated, giving a semi-solid (1·286 g.). This was dissolved in alcohol and titrated with alcoholic potash and then saponified with excess of this reagent. (Found: carboxyl equiv. 213 and ester equiv. 216; $HOOC.(CH_2)_7COOEt$ has mol. wt. 205). The solution was acidified (HCl), cooled and filtered, yielding crude azelaic acid (0·641), m.p. 102·5–103°. The azelaic acid was recrystallized once from hot water, when it had m.p. 107·5–108° alone, or mixed with authentic azelaic acid (m.p. 106·5–108°).

The ethereal solution of the distillate was titrated, with shaking, in a separating funnel with *N* NaOH. No pure product was isolated from the neutral ether; a carbonyl group was present since reaction occurred with 2:4-dinitrophenylhydrazine but no identifiable hydrazone was isolated; a semicarbazone could not be formed.

The aqueous solution gave a sodium salt (0·875 g.), equiv. 135. This was dissolved in water (55 ml.), acidified (H_2SO_4) and distilled. The first 20 ml. and the next 29 ml. of distillate gave Na salts, 0·380 g., equiv. 136, and 0·317 g., equiv. 132, respectively. The former was converted into its *p*-bromophenacyl derivative which had m.p. 71·5–72° and mixed m.p. 71–72° with authentic *p*-bromophenacyl caproate (m.p. 71·5–72°).

SUMMARY

1. The results of ozonolysis and of acetone-$KMnO_4$ oxidation of methyl arachidonate are in agreement with the constitution (II) $\Delta^{5.8.11.14}$-eicosatetrenoic acid, for arachidonic acid, which was proposed by Dolby *et al.* [1940].

2. Contrary to the results of Takahashi [1921] ozonolysis of ethyl linoleate yields products in accordance with the generally accepted structure for linoleic acid, $\Delta^{9.12}$-octadecadienoic acid (IV).

We desire to express our gratitude to Prof. D. Keilin for hospitality at the Molteno Institute and to Dr F. G. Mann for permission to use the ozonolysis apparatus in the University Chemical Laboratory, Cambridge.

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Studies on Diffusing Factors

10. NOTE ON THE FORMATION OF VISCOUS MATERIALS BY *CLOSTRIDIUM BUTYLICUM*

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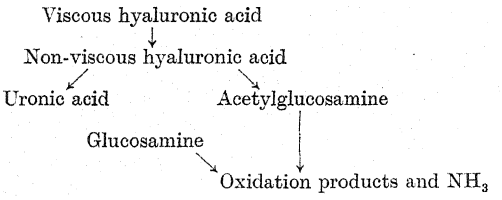
The enzyme system known as hyaluronidase, which exists in some tissues and bacteria, hydrolyses polysaccharides of the hyaluronic acid type. Hyaluronic acid is built up from equimolecular amounts of acetylglucosamine and uronic acid residues [Meyer, 1938]. In the hyaluronidase complex two well-defined individual components have so far been detected [East, Madinaveitia & Todd, 1941]. One of them reduces the viscosity of the viscous polysaccharide without liberating any appreciable amount of reducing sugar. The other is a glucosaminidase which does not attack the viscous polysaccharide but which may play a role in the later stages of the hydrolysis of the polysaccharide by splitting glucosaminic linkages. It is possible that a third component also exists, a uronidase which would hydrolyse the uronidic linkages of the polysaccharide. No evidence has so far been obtained for the presence of such an enzyme in the preparations assayed. The substrate used was menthol glucuronide.

The presence of diffusing factors has been demonstrated in all the sources of hyaluronidase as yet known (e.g. in mammalian testicle, leech extract, snake venoms and some bacteria). It has been claimed that hyaluronidase and diffusing factor are the same substance [Chain & Duthie, 1940; McClean, 1941*b*; McClean & Hale, 1941]. There is, however, a discrepancy between the viscosity-reducing power

and the diffusing potency of various samples assayed [Madinaveitia, Todd, Bacharach & Chance, 1940]. There is no relation at all between the diffusing potency and glucosaminidase activity.

Mammalian testicle possesses, besides hyaluronidase, an enzyme system which deaminates and oxidizes amino sugars [Lutwak-Mann, 1941]. This enzyme system is also present in kidney, brain and in some bacteria. In testicle we find a complete mechanism to utilize the glucosamine present in hyaluronic acid according to the following scheme:



The components of this system which hydrolyse hyaluronic acid have been detected in culture filtrates of bacteria that produce diffusing factor. In this connexion the work of McClean [McClean & Hale, 1941; McClean, 1941*b*] is perhaps significant. He has found that inclusion of hyaluronic acid in the culture media of *Clostridium welchii* and some strains of *Streptococcus* and *Pneumoccocus* enhances the formation of both diffusing factor and hyaluronidase. In the case of *Clostridium welchii* it also increases bacterial growth to about the same extent as glucose. It is of interest to note that no N-acetyl glucosamine could be detected in the medium supplemented by hyaluronic acid in which *Cl. welchii* had grown [McClean & Hale, private communication]. This indicates that the micro-organism has enzymes which, besides hydrolysing hyaluronic acid, also utilize the free glucosamine thus formed. McClean [1941 *a*, *b*] has also observed that addition of hyaluronidase may modify the cultural characteristics of some bacteria. Certain strains of *Streptoccoccus* which in young culture have capsules become non-capsulated when grown in the presence of hyaluronidase. The addition of hyaluronidase to a capsulated strain immediately destroys the capsules. From these capsulated strains a polysaccharide has been isolated [Kendall, Heidelberger & Dawson, 1937] which is similar, or perhaps identical with hyaluronic acid and McClean has found that formation of capsules and production of hyaluronidase do not coexist in the strains examined. In the case of *Pneumococcus* the polysaccharide from which the capsules are formed [Heidelberger, Kendall & Sherp, 1936] although similar, is not identical with hyaluronic acid, and accordingly inclusion of hyaluronidase in the culture medium does not affect capsulation.

The production of polysaccharides by micro-organisms is by no means always connected with capsule formation. A number of bacteria, for example, elaborate viscous substances which they secrete into the culture medium. Whilst conducting biotin assays in connexion with other work in this laboratory, using as test organism *Cl. butylicum* [Snell & Williams, 1939; Peterson, McDaniell & McCoy, 1940] one of us observed that this spore-bearing anaerobe makes the medium in which it is growing viscous. *Cl. butylicum* grows in relatively simple glucose media using asparagin as the only source of nitrogen. Biotin is an essential factor for its growth; this can be supplied either as the pure substance [Snell & Williams, 1939; Woolley quoted by du Vigneaud, Hofmann & Melville, 1941] or in the form of peptone [McDaniell, Woolley & Peterson, 1939].

Since certain of the cultural characteristics of this micro-organism are fairly easily reproducible, it seemed of interest to investigate the factors affecting the production of viscous materials. While we have not been able to obtain evidence for the presence of glucosamine in the viscous material, nor is the viscosity of the latter destroyed by hyaluronidase, yet the growth of *Cl. butylicum* and the formation of viscous material are both inhibited to an appreciable extent by growing the organism in the presence of purified hyaluronidase preparations.

The formation of viscous materials is, however, much more markedly affected by other factors such as the initial concentration in the medium of glucose and of the Bactopeptone which was used in our experiments as a source of biotin. There are optimal concentrations of both glucose and peptone for bacterial growth and for production of viscous materials (Fig. 1). At any given concentration of peptone the optimal concentrations of glucose to obtain maximal growth and maximal formation of viscous materials are not very different. However, if the amount of sugar is constant the concentration of peptone which leads to the formation of most viscous material is much lower than that which produces maximal growth. At the highest concentrations of peptone investigated, practically no viscous material was formed, irrespective of the amount of sugar present, and yet the optimal concentration of peptone for bacterial growth had not been reached. This cannot be attributed to all the glucose being metabolized by the bacteria; at high concentrations of glucose a large proportion of the sugar remained unused.

EXPERIMENTAL

Influence of hyaluronidase on the growth of Cl. butylicum. Aseptic conditions were observed throughout this work.

The strain of *Cl. butylicum* used (kindly supplied by Prof. Elizabeth McCoy, Wisconsin, U.S.A.) had been kept for some time in the form of dry spores.

The preparation of hyaluronidase used was prepared from aqueous extracts of bulls' testicle by Pb acetate precipitation followed by $(NH_4)_2SO_4$ fractionation and dialysis [Madinaveitia, 1941]. The solutions were sterilized by filtration through a Seitz filter.

A series of tubes each having 15 ml. medium containing 1 % bactopeptone, 2 % glucose, 0·2 % asparagine, Speakman's [1923] salt mixture and a trace of reduced iron was prepared. Five of the tubes to be used as controls received 1 ml. of a hyaluronidase solution, 10 mg./ml., before being sterilized in the autoclave. It is known that hyaluronidase is thermolabile. Another five tubes received the hyaluronidase after they had been sterilized. All tubes were inoculated with a loopful of a 24 hr. old culture and incubated in a McIntosh and Fildes jar for 60 hr. at 37°. Then the contents of the tubes were centrifuged until clear. The precipitate was washed with water and the washings rejected. The N content of the washed precipitate was determined and this 'Bacterial N' assumed to be a measure of the number of bacteria present. The supernatant was heated 5 min. at 100° and made clear again by filtration through paper. The viscosity was now determined in an Ostwald viscometer (temp. 25°). The reducing material in the supernatant, as determined by the method of Bertrand, was assumed to be the unused glucose. The figures obtained are given in Table 1.

Table 1

	Bacterial N mg./ml.	Specific viscosity	% sugar consumed
Medium + inactivated hyaluronidase (control)	1·75	0·71	87
	1·68	0·79	92
	1·85	0·93	100
	1·86	0·87	97
	2·01	0·77	97
Medium + hyaluronidase	1·23	0·43	67
	1·29	0·46	79
	—	0·44	79
	1·29	0·51	75
	1·18	0·50	94

Influence of glucose and peptone on the growth of Cl. butylicum. To obtain an indication of the time of incubation required to attain maximal growth, the following experiment was carried out: A series of tubes containing 10 ml. medium (2 % A.R. glucose, 0·6 % Bactopeptone, 0·2 % asparagine, Speakman's salt mixture and reduced iron) was inoculated with a loopful of a 24 hr. old culture of *Cl. butylicum*. All tubes were incubated at 37° in the same McIntosh and Fildes jar. Lots of three tubes were removed from time to time and anaerobiosis re-established before proceeding with the incubation. As soon as the tubes were removed the culture was centrifuged and the 'Bacterial N', specific viscosity and the amount of glucose consumed were estimated as previously indicated. The results obtained are recorded in Table 2; mean values are printed in italics.

Table 2

Time of incubation hr.	Specific viscosity	% glucose consumed	Bacterial N mg./10 ml.
14	0·02, 0·04, 0·04	7·8, 11	0·35, 0·62, 0·60
	0·03	*9·4*	*0·52*
21	0·22, 0·22, 0·45	32·5, 37, 39	0·62, 0·69
	0·30	*36·2*	*0·65*
40	1·15, 1·06, 2·69*	85, 92, 77	0·75, 1·03, 1·11
	1·10	*85*	*0·96*
99	1·50, 1·18, 1·48	96, 99·5, 97	1·15, 1·33, 1·08
	1·39	*97·5*	*1·20*

* Not included in the mean.

From Table 2 one can see that the lag phase is about 10 hr. and that the end of the logarithmic phase is reached in about 2 days.

To determine the influence of glucose and of peptone on the growth of *Cl. butylicum* a series of tubes containing 10 ml. medium of various concentrations of Bactopeptone and of glucose was prepared. They all had 0·2 % asparagine, Speakman's salt mixture and reduced iron. They were sterilized, inoculated, and after 70 hr. incubation at 37°, the bacterial N, viscosity and amount of sugar used were determined. All these operations were carried out as previously described. The concentrations of glucose and of peptone which were examined, and whether or not growth was observed in each of the tubes, are recorded in Table 3.

Table 3

Bacto-peptone mg./ml.	Glucose mg./ml.						
	20	30	50	60	80	150	300
2·5	+	.	+	.	.	−	−
5	.	+	.	+	+	.	.
10	.	+	.	+	+	.	.
12·5	+	.	+	.	.	−	−
20	.	+	.	+	+	.	.
25	+	.	+	.	.	−	−
30	.	+	.	+	+	.	.
40	.	+	.	+	+	.	.
50	+	.	+	.	.	−	−

+ =growth; − =no growth.

Fig. 1 is a graphic representation of the values obtained for bacterial N, specific viscosity and glucose consumed. It is a contour map of the surfaces resulting from plotting these values along an axis perpendicular to the plane of the paper. On this plane the concentrations of glucose and of peptone are represented.

SUMMARY

Cl. butylicum produces viscous materials when grown in a glucose-asparagine-mineral salts medium containing Bactopeptone as a source of biotin. Both the growth and the production of viscous polysaccharides are inhibited to a certain extent by inclusion of hyaluronidase concentrates in the medium.

The effect upon some cultural characteristics of *Cl. butylicum* of the initial concentrations of glucose and peptone in the medium has been studied.

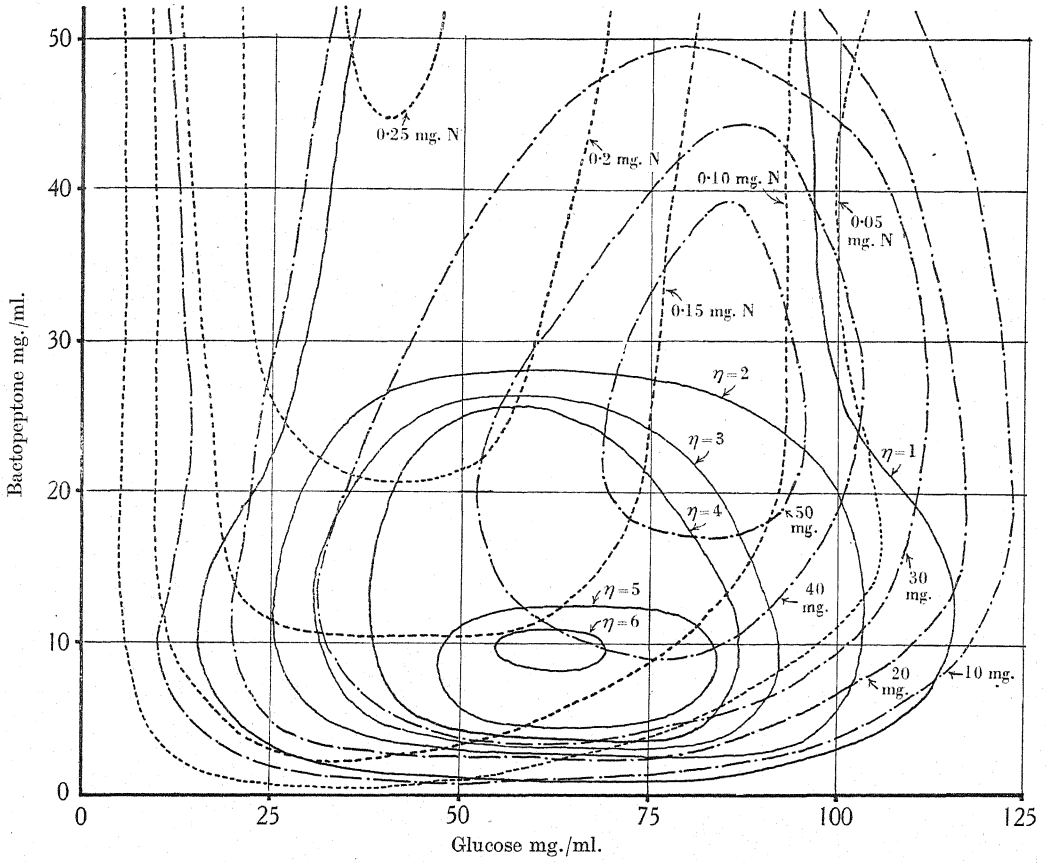


Fig. 1. Effect of glucose and Bactopeptone on *Cl. butylicum*. —— Viscosity of medium. - - - - - Bacterial N (mg./ml.). ·—·—·—· Glucose consumed (mg./ml.).

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The 'Catalase Test', with Special Reference to *Acetobacter* Species

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The first statement on the occurrence of catalase in bacteria seems to have been made by Beijerinck [1901], who pointed out that whereas the lactobacilli do not contain this enzyme *Acetobacter* species show a positive reaction for catalase. Callow [1923] compared 9 anaerobes and 12 aerobes (none of the aerobes were *Acetobacter* species) with respect to catalase content and noted that the anaerobes were catalase negative and the aerobes were catalase positive. Kluyver [1924], discussing the catalase of micro-organisms, concluded provisionally that this enzyme occurs only in those bacteria which use free oxygen in their metabolic processes. Further information on catalase in bacteria was provided by McLeod & Gordon [1923] and by Virtanen & Karström [1925]. Morgulis, Beber & Rabkin [1926], in their quantitative studies of catalase activity, observed that the activity, which was greatest at 0–10°, diminished with increasing experimental temperatures. The same authors also found that exposure of catalase to temperatures up to 40° for 1 hr. caused practically no inactivation, and cooling the enzyme did not reduce its activity until −10° was reached. The rate of destruction of catalase increased not only with temperature but also with concentration of H_2O_2, and the destruction followed the course of a monomolecular reaction. Experiments with various H_2O_2 concentrations at different temperatures showed that the maximum activity invariably occurred with 0·54*N* H_2O_2. Using catalase from beef kidney they found its activity to be strong over the range *p*H 5–9 with greatest activity between *p*H 6 and 8. The activity was entirely lost at *p*H 2.

Since Beijerinck's early discovery of the presence of catalase in *Acetobacter* species there have been no further reports on the catalase activity of these particular organisms apart from those of Visser't Hooft [1925, 1939], who recorded the presence of this enzyme in *A. aceti*, *A. rancens*, *A. xylinum*, *A. melanogenum*, and *A. suboxydans*, and who described *A. peroxydans*, a species devoid of catalase. No other catalase-negative acetic-acid bacteria have since been reported.

In recent work with 11 species of *Acetobacter* we observed that very erratic results were obtained when cultures were subjected to the 'catalase test'. When well-developed colonies which had been growing at 25° on malt-extract agar (*p*H 5·0) in Petri dishes for 14 days were treated with 0·5*N* H_2O_2, three species gave no response and some of the others reacted only weakly. The results obtained with cultures 8 days' old under conditions which otherwise were identical were practically the same (Table 1).

Table 1

Species of *Acetobacter*	Reaction with H_2O_2 given by cultures aged		*p*H developed by the several species when grown in malt extract (initial *p*H 5·0) at 25° for	
	8 days	14 days	3 days	7 days
(1) *A. aceti*	−	−	3·85	3·60
(2) *A. acetosum*	+	+	3·60	3·25
(3) *A. kuetzingianum*	+	+ +	4·60	4·30
(4) *A. pasteurianum*	+ +	+ +	4·55	4·05
(5) *A. suboxydans*	− ?	−	4·25	3·70
(6) *A. xylinum*	+ + +	+ + +	4·15	3·50
(7) *A. gluconicum*	+	+	4·20	3·75
(8) *A. ascendens*	+ +	+ +	4·60	3·90
(9) *A. capsulatum*	+ ?	−	3·05	2·70
(10) *A. acetigenum*	+ +	+ +	3·80	3·35
(11) *A. turbidans*	+ +	+ +	3·50	3·45

The particular degree of catalase activity shown by each of the several species is probably the resultant of two factors, namely, the quantity of catalase made available by the cells, and the hydrogen-ion concentration produced in the medium by the activities of the cells. Thus, for example, in the cases of the species numbered (4), (6) and (10), which were strong growers and proliferated freely on the slopes, it is probable that the total catalase available was relatively great in amount, hence the influence of the moderate fall in *p*H value was not so obvious and the cultures reacted in a definitely catalase-positive manner. In the case of the species numbered (1) and (5) where growth was relatively scanty, the moderate fall in *p*H had a much greater effect and the cultures proved catalase-negative. Finally, in the case of species number (9), although growth was very abundant it was accompanied by a very considerable fall in *p*H value and the catalase test gave first a doubtful and later a negative result.

The different species were now inoculated into tubes of sterile malt extract (sp.gr. 1·036) buffered with 3 % of $CaCO_3$ which had brought the *p*H to 6·01. After 3 days at 25°, growth was evident in all the tubes, and at this stage in every case the *p*H value

lay between the limits 5·56 and 5·97. Hence such a medium is a satisfactory one for the control of the acidity developed by *Acetobacter* species. Slopes were then prepared from similar buffered malt extract to which 1·5 % of agar had been added. Before addition to the extract the $CaCO_3$ had been sterilized in steam and afterwards heated at 160° for 2 hr. to ensure that resistant spore-forming organisms were killed. Cultures grown on these slopes at 25° were all found to give a positive catalase reaction on the 3rd day after inoculation and all still reacted positively on the 14th day.

The following experiment was then undertaken. Malt extract agar, buffered with $CaCO_3$, was poured in separate 10 ml. portions into a set of conical flasks (250 ml.) and sterilized in the usual manner. These flasks were inoculated with the several *Acetobacter* species. In each case a malt-extract culture grown for 3 days at 20° provided the inoculum, 1 ml. quantities being run from sterile pipettes into the several flasks and spread over the surface of the agar by suitable manipulation of each flask. These flask cultures were incubated at 20° for exactly 3 days and estimations of catalase activity were then performed. Into each flask, including an uninoculated 'blank', there were poured exactly 50 ml. of 0·5*N* H_2O_2 (adjusted to *p*H 6·0) and estimations of H_2O_2 were made immediately, then after 15 min., 1 hr., 2 hr., and 3½ hr. These were done volumetrically by the method given by Kolthoff & Sandell [1937]. In the case of the 'blank' the loss (about 10 %) of H_2O_2 which was observed up to 45 min. was probably due not to decomposition but to adsorption, but in all the culture flasks much greater losses of H_2O_2 were found to have occurred (Table 2).

Table 2

Acetobacter species	% of added H_2O_2 destroyed after min. 15	60	120	210
A. aceti	19·0	23·5	25·0	25·0
A. acetosum	26·0	29·5	30·0	31·0
A. kuetzingianum	16·0	20·5	21·0	21·5
A. pasteurianum	10·5	35·0	35·5	37·0
A. suboxydans	16·5	30·5	32·5	32·5
A. xylinum	11·5	14·5	16·5	16·5
A. gluconicum	18·5	28·5	30·0	30·0
A. ascendens	12·5	18·0	20·5	21·5
A. capsulatum	23·5	40·0	59·0	75·0
A. acetigenum	10·0	16·5	19·5	19·5
A. turbidans	26·0	36·0	42·0	45·0
'Blank'	7½	15	30	45
Uninoculated medium	3·5	5·5	9·0	10·5

Note. A final set of estimations carried out at 20 hr. from the time of addition of H_2O_2 showed that *A. capsulatum* had destroyed 83·0 % and *A. turbidans* 47·0 % of the added H_2O_2.

If the percentages of H_2O_2 destroyed are plotted against the time, the relative catalase activities of the organisms which were grown on the buffered medium in the flasks are indicated very clearly and the curves conform well to the shapes of the curves of catalase activity obtained from estimations conducted by Morgulis *et al.* [1926] who used liver catalase. The relatively high degree of catalase activity shown by both *A. capsulatum* and *A. turbidans* is noteworthy; these are two new species both isolated from sour beers and described, respectively, by Shimwell [1936] and by Cosbie, Tošić & Walker [1942]. From this work the following general conclusions can be drawn relating to the catalase activity of *Acetobacter* species: the acid produced by *Acetobacter* species in a medium containing malt extract inactivates catalase. Young cultures which have not passed beyond the early logarithmic phase of growth show a positive catalase reaction even in unbuffered media, because formation of acid does not take place to an appreciable extent until the organisms are well advanced in logarithmic growth. (An exception is *A. peroxydans* which, as mentioned earlier, is catalase-negative.) Older cultures should not be examined for catalase activity unless grown in a buffered medium similar to that described in this paper. The test should be performed as soon as growth has become well established.

Before examining, for catalase activity, new organisms other than *Acetobacter* species, it would seem desirable, in view of our observations, first to determine whether the organisms in question form acid in the medium used and, if they do so, then the cultures prepared for the catalase test should be cultivated in the presence of a suitable buffering agent. It is also necessary to bear in mind that the rate of loss of catalase activity increases with rise in temperature. Hence the cultures prepared for the test should be incubated at the lowest point of the optimum temperature range for that particular micro-organism.

Finally, it may be remarked that the inactivation of catalase by acid as described in this communication is an interesting parallel to the observation of Butlin [1938] that *A. suboxydans* contains as one of its enzyme components an acid-sensitive system which catalyses a reaction in which the O_2 absorbed is equivalent to the CO_2 evolved. In certain conditions this organism cannot exercise this more highly oxidizing function because of the conditions it creates in the cultures if they are unbuffered (e.g. primary production of gluconic acid can occur in a glucose medium of *p*H 6·0 by the agency of another oxidizing enzyme present in *A. suboxydans* and this gluconic acid inhibits the first-mentioned oxidizing system).

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Riboflavin, Vitamin B_6 and Filtrate Factors in Wheaten Flours and Offals

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In recent years there have been many studies of the vitamin B_1 content of wheaten flours and breads, but relatively few estimations have been made of the content of members of the vitamin B_2 complex. Some five years ago Copping & Roscoe [1937] published a survey of the water-soluble B vitamins in yeast, flour and bread, and gave values for the riboflavin and 'factors other than flavin'. These estimations were, however, made at a time when our knowledge of the members of the vitamin B_2 complex was more incomplete than it is at present, and the methods used appear far from satisfactory in the light of later work. New methods for the biological estimation of riboflavin have been described by El Sadr, Macrae & Work [1940], and on the basis of these methods others have now been evolved for estimating vitamin B_6 and filtrate factors. Almost all of the previous values for riboflavin and vitamin B_6 which have been put forward for flours have been obtained by chemical or microbiological methods.

The term filtrate factors is taken to mean all growth factors of the vitamin B_2 complex required by the rat, other than riboflavin, nicotinic acid and vitamin B_6 [El Sadr, Hind, Macrae, Work, Lythgoe & Todd, 1939; Macrae, Todd, Lythgoe, Work, Hind & El Sadr, 1939]. Filtrate factors are now known to include pantothenic acid, biotin and one or more unidentified substances. Lepkovsky [1942] reviews the subject most lucidly.

It is important at the present time to have some precise indication of the nutritive value of the flour and bread available to the people of this country, and on this account, as well as to obtain values by biological methods for comparison with those obtained by other methods, the further study of wheaten flours was undertaken.

MATERIALS

The materials used were supplied through the courtesy of the Research Association of British Flour Millers, who arranged to have suitable flours milled and to store some of the grist so that further samples could be milled if required. In January 1941 the following samples were milled and put into store in a cool place in airtight containers: (1) straight run unbleached white flour of 73 % extraction; (2) wholemeal flour of 100 % extraction; (3) weatings or middlings; (4) bran; (5) germ. The germ represented all the clean germ obtained in the milling of the white flour, and the bran and weatings together comprised the balance of the wheat after removal of 73 % as white flour.

About 6 months later, when the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute had laid down the specification for the preparation of the 85 % extraction national wheatmeal [Medical Research Council, 1941], a sample of this type of flour was milled from the original grist and tested in the same way as the other samples.

Table 1. *Composition of the flours and offals* (%)

	Water*	Ash	Nitrogen	Protein
73 % flour	13·6	0·411	2·042	11·64†
85 % flour	12·64	0·861	2·103	11·98†
100 % flour	13·53	1·545	2·190	12·48†
Weatings	14·6	4·31	3·04	17·33†
Bran	13·9	6·55	2·495	15·6‡
Germ	13·98	4·18	4·76	29·75‡

* Dried at 105–110°. † N × 5·7. ‡ N × 6·25.

The vitamin estimations were made on the flours as they were received; they contained about 13 % of water (Table 1).

Since the earlier results of Copping & Roscoe [1937] had shown that there was no great difference in the amounts of vitamin B_2 factors present in flours and in the breads baked from them, it was considered unnecessary to bake breads from the flours used for the present investigation, and estimations were made only on the flours.

METHODS

In all tests the criterion was the rate of growth of young rats weaned at 21 days and given the basal diet immediately. In each test comparison was made with a standard preparation of the vitamin in question. Littermate rats, as far as possible of the same sex, were given the doses to be compared, so that for each group of four or more animals on a dose of the cereal product there was a corresponding group on a dose of the standard [Coward, 1938].

The rats received a basal diet containing 60 % carbohydrate (maize starch or cane sugar), 20 % purified casein, 15 % fat and 5 % McCollum's normal salt mixture. The fat consisted of 4 parts of hardened arachis oil and 1 part of pure lard. When maize starch was the source of carbohydrate the dry ingredients were mixed with their own weight of water and the mixture was steamed for 3–5 hr., depending on the amount made up, in order to dextrinize the starch and guard against refection [Fridericia, Freudenthal, Gudjonsson, Johansen & Schoubye, 1927]. The sugar-containing diet was not cooked.

The flours tested were made up into similar diets containing 68 % flour, 12 % casein, 15 % fat and 5 % salt mixture, and these were cooked in the steamer in the same way as the basal diet. These diets contained approximately the same proportions of carbohydrate, protein and fat as the basal diet and were given as daily doses in weighed amounts, the remainder of the animal's food requirement being made up by giving the basal diet *ad lib.* In the case of bran, difficulty was experienced in making a cohesive mixture with a high proportion of bran in the diet, and a diet containing 30 % bran, 30 % maize starch, 20 % casein, 12 % arachis oil, 3 % lard and 5 % salt mixture was used. The doses of test diets were given in amounts varying from 3 to 10 g. daily, and if any of the test diet was left an equivalent extra amount was given on the next day. As the diets were mixed with their own weight of water the intake of flour was calculated as 34 % of the diet eaten. Thus a daily dose of 10 g. of the test diet was equivalent to an intake of 3·4 g. of the flour. Wheat germ was a sufficiently rich source of all the factors tested to be given alone as a separate dose apart from the diet.

Riboflavin estimation. The method was essentially that of El Sadr *et al.* [1940]. The basal diet containing maize starch was supplemented with 0·1 ml. cod liver oil as a source of vitamins A and D, with pure vitamin B_1, and with a whole-liver extract, treated with charcoal to remove the riboflavin, as a source of all the vitamin B_2 factors except riboflavin. Norite charcoal, as used in the original method, was no longer available, but a decolorizing charcoal was substituted and, after some adjustment of quantities, was found satisfactory. In every test two doses of pure riboflavin were given as standard for comparison, usually 6 and 12 μg. per day. The test materials were fed at two levels also, and 4–8 rats were used for each dose level. In the tests with white flour the riboflavin content was so low that the response was too poor to obtain a result of any accuracy; 6 μg. riboflavin were therefore given daily in addition to the flour dose, and the value of the latter deduced by calculation from the curve of response.

Vitamin B_6 (pyridoxine) estimation. The method for the biological estimation of vitamin B_6 was based on that for riboflavin. Rats taken at weaning and weighing about 40 g. were given the vitamin B-free basal starch diet with the addition of daily doses of vitamin B_1 and cod liver oil for 7 days. They were then transferred to a basal diet containing sugar and given additional daily supplements of 40 μg. riboflavin and a fuller's earth filtrate from a liver residue extract. This filtrate supplied the filtrate factor portion of the vitamin B_2 complex; a dose of 1 ml. was equivalent to 6 g. of the original liver. It was found unsatisfactory to give a starch diet in tests for vitamin B_6, as all cereal starches contain some of this vitamin [Chick, Macrae & Worden, 1940]. After some preliminary experiments it was found satisfactory to give the basal diet and supplements other than vitamin B_6 for a standard period of 14 days before administering the test material. The rats usually grew well in the 1st week, but very poorly in the 2nd week, and showed a satisfactory response to a subsequent adequate dose of vitamin B_6. Two levels of dose of pure crystalline vitamin B_6 hydrochloride were given in every test, and the test material was fed at two levels as in the riboflavin tests. This permitted of a good comparison in most cases, since flours and offals were good sources of vitamin B_6.

Estimation of filtrate factors. The filtrate factor portion of the vitamin B_2 complex is known to contain pantothenic acid and at least one other factor [Lythgoe, Macrae, Stanley, Todd & Work, 1940]. An attempt was made to estimate pantothenic acid separately, but so far difficulty has been experienced in devising a basal diet free from pantothenic acid yet containing all other factors of the vitamin B_2 complex. It has, therefore, been possible

only to test for the whole filtrate complex. For this purpose young rats were prepared as for the vitamin B_6 tests and were given the basal sugar diet supplemented with 10 μg. crystalline vitamin B_1, 40 μg. riboflavin, 10 μg. crystalline vitamin B_6 hydrochloride, 1 mg. inositol, 1 mg. nicotinic acid, 3 mg. choline chloride and 0·1 ml. cod-liver oil daily. It was thought advisable to give the additional supplements of inositol, nicotinic acid and choline chloride when the other B_2 vitamins were supplied in the crystalline form. Choline chloride was certainly needed, for it was observed in some tests, before we had seen the reports of György & Goldblatt [1940], that the young rats occasionally died without apparent cause within a week of receiving the basal diet with vitamin B_1, riboflavin and vitamin B_6. Abnormalities of the liver and kidneys, as described by György & Goldblatt, were found in these animals at autopsy, and after choline chloride was given no further losses occurred. As in the vitamin B_6 tests a preliminary period of 14 days with all vitamin B supplements other than filtrate factors was found suitable as a means of preparing the rats for testing. As no source of filtrate factors in a pure form was available a dried baker's yeast was taken as standard of comparison in these tests; it was given at two levels of 100 and 200 mg. daily. The results were calculated in terms of mg.-equivalents of the standard yeast.

Table 2. *Summary of tests for riboflavin content of flours and offals*

Supplement	Daily dose	No. of rats	Av. weekly gain in wt. g.	Calc. content of riboflavin μg./g. fresh wt
1. White flour: 73% extraction	2·7 g. + 6 μg. riboflavin	14	14	0·85
Riboflavin	6 μg.	14	12	
	12 μg.	14	16	
2. National wheatmeal: 85% extraction	1·7 g.	8	7·5	2·0
	3·4 g.	8	10	
	2·7 g. + 6 μg. riboflavin	4	16	
Riboflavin	6 μg.	12	11	
	12 μg.	12	15	
3. Wholemeal: 100% extraction	2·0 g.	4	10	2·3
	4·0 g.	4	14	
	2·7 g. + 6 μg. riboflavin	4	17	
Riboflavin	6 μg.	8	12	
	12 μg.	8	16	
4. Weatings	1·7 g.	4	14	4·0
	2·7 g.	4	15	
Riboflavin	6 μg.	4	13	
	12 μg.	4	16	
5. Bran	1·6 g.	3	14	4·8
	2·16 g.	5	18	
Riboflavin	12 μg.	4	18	
	18 μg.	4	21	
6. Germ I	200 mg.	4	9	7·8
	400 mg.	4	14	
Riboflavin	6 μg.	4	16	
	12 μg.	4	19	
7. Germ II	200 mg.	4	12	12
	400 mg.	4	13	
Riboflavin	6 μg.	3	16	
	12 μg.	3	20	

Table 3. *Summary of tests for vitamin B_6 content of flours and offals*

Supplement	Daily dose	No. of rats	Av. weekly gain in wt. g.	Calc. content of vitamin B_6 hydrochloride μg./g. fresh wt.*
1. White flour: 73% extraction	1·7 g.	8	11·5	2·5
	3·4 g.	8	15·5	
Vitamin B_6 hydrochloride	2·5 μg.	8	10·5	
	5·0 μg.	8	13	
2. National wheatmeal: 85% extraction	1·36 g.	8	14	4·2
	2·7 g.	8	18	
Vitamin B_6 hydrochloride	2·5 μg.	8	9	
	5·0 μg.	8	13·5	
3. Wholemeal: 100% extraction	1·0 g.	8	14	*c.* 5·0
	2·0 g.	8	19	
Vitamin B_6 hydrochloride	2·5 μg.	3	12	
	5·0 μg.	8	13·5	
	10 μg.	4	14·5	
4. Weatings	1·02 g.	4	20	13
	2·04 g.	4	27	
Vitamin B_6 hydrochloride	2·5 μg.	4	9	
	5·0 μg.	4	14	
5. Germ I	200 mg.	4	12	13·6
	400 mg.	4	15	
Vitamin B_6 hydrochloride	2·5 μg.	3	11	
	5·0 μg.	4	15	
6. Germ II	200 mg.	4	9·5	12
	400 mg.	4	15	
Vitamin B_6 hydrochloride	2·5 μg.	4	10·5	
	5·0 μg.	4	14·5	

* These values $\times 0{\cdot}82$ give the actual vitamin B_6 content of the materials.

All results given in the final columns of Tables 2, 3 and 4 are calculated by reference of the weight increase corresponding to the test doses of the unknown substances, to the straight line obtained by plotting the logarithm of the two standard doses to the growth response which they elicited. In no case was use made of a composite curve constructed for the responses to the standard, but in each separate experiment the results were calculated with reference to the line obtained from the results with the standard doses in that particular test.

Table 4. *Summary of tests for filtrate factor content of flours and offals*

Supplement	Daily dose	No. of rats	Av. weekly gain in wt. g.	Calc. content of filtrate factors mg.-equiv. of D.C.L. yeast per g.
1. White flour: 73% extraction	1·7 g.	8	7·5	40–50
	2·7 g.	8	11	
D.C.L. yeast	100 mg.	8	9	
	200 mg.	8	12	
2. National wheatmeal: 85% extraction	1·36 g.	8	10·5	80
	2·7 g.	8	16·5	
D.C.L. yeast	100 mg.	7	10	
	200 mg.	8	15·5	
3. Wholemeal: 100% extraction	1·02 g.	11	11	110
	2·04 g.	11	16·4	
D.C.L. yeast	100 mg.	10	9·7	
	200 mg.	11	15·5	
4. Weatings	1·02 g.	4	15	190
	2·04 g.	4	21	
D.C.L. yeast	100 mg.	4	8	
	200 mg.	4	15	
5. Germ I	250 mg.	4	6	200
	500 mg.	4	10	
D.C.L. yeast	100 mg.	4	11	
	200 mg.	4	17	
6. Germ II	250 mg.	4	5·5	250
	500 mg.	4	9	
D.C.L. yeast	100 mg.	4	8	
	200 mg.	4	14·5	

DISCUSSION

The recorded estimations of riboflavin in cereals include few carried out by biological methods with rats as the test animals. The earlier studies of Copping [1936] and Copping & Roscoe [1937] gave an approximation, but no quantitative values, since factors other than riboflavin were limiting the growth of the rats under the conditions of the tests. Most of the existing values for riboflavin have been obtained by fluorimetric or microbiological methods. Conner & Straub [1941*a*] have reported good agreement in their results obtained by a combined method for estimating vitamin B_1 and riboflavin simultaneously, and by a simple fluorimetric method for estimating riboflavin alone, as used by Hodson & Norris [1939]. Hodson & Norris compared the results obtained with their fluorimetric method for estimating riboflavin with those obtained by the microbiological method of Snell & Strong [1939] and also found good agreement.

All the values reported for wheat and wheat products by the above workers are considerably lower than those found in the present biological estimations. Dr Davis and Mr Newland, of the National Institute for Research in Dairying, Shinfield, have tested the present series of flour samples by a modification of the microbiological method of Snell & Strong and have obtained results in remarkably good agreement with those obtained in the present biological tests (Table 5). Barton-Wright [1942], who used the microbiological method, reports a series of values in good agreement with those obtained by Dr Davis and Mr Newland and by the biological method. Andrews, Boyd & Terry [1942] described studies of riboflavin in cereals by the microbiological method, but their results were much lower than those obtained at Shinfield. It may be that the wheat blend used in milling the present series of flour samples was particularly rich in riboflavin, though this is unlikely. Conner & Straub [1941*a*] report values ranging from 0·89 to 1·91 μg. per g. for 15 varieties of hard wheats and from 0·81 to 1·48 μg. for 16 varieties of soft wheats. The generally higher results obtained by biological methods with rats, as compared with those obtained by fluorimetric or microbiological methods may be due to the difficulty in the extraction of riboflavin from cereal products which is necessary in the latter procedures, or, on the other hand, to the presence of other factors affecting the growth of rats in the biological method. The biological method for estimating riboflavin as originally described by El Sadr *et al.* [1940] has been applied to examining many materials in this laboratory and has been constantly checked by experiments with different doses of pure riboflavin. No evidence has been obtained of the need of the rat for B vitamins other than those present in the liver extract used. The good agreement between the results of biological and microbiological tests on the materials used in this study would also appear to confirm the reliability of the biological method.

Fraenkel & Blewett [1941] demonstrated differences in the riboflavin content of patent, straight run and wholemeal flours by a method in which the criterion adopted was the development of the flour beetle, *Tribolium confusum*. These workers have also demonstrated graduation of riboflavin content in the flour samples used in the present study but have not as yet been able to make a quantitative estimation by their method of riboflavin in such relatively poor sources.

For comparison the riboflavin values obtained for different wheat products by several observers are collected together in Table 5.

Values for the vitamin B_6 content of cereals have not yet been reported to any extent, though it was demonstrated qualitatively some years ago [Birch & György, 1935; Copping, 1936] that most cereals and their milling products were good sources of this nutrient. Swaminathan [1940 *a*, *b*] developed a

Table 5

Material	Riboflavin μg./g. estimated by (1)	(2)	(3)	(4)	(5)	(6)
73% ext. flour	0·85	0·83	0·75–1·25	0·62	0·4	—
85% ext. flour	2·00	2·14	2·00–2·1	—	—	—
100% ext. flour	2·30	2·78	2·65–3·7	1·00	—	0·89–2·03
Weatings (middlings)	4·00	5·90	—	3·80	1·3	—
Bran	4·80	—	5–8	2·80	1·0	3·17
Germ	7·8 and 12	—	9·7–12·85	—	—	3·78–5·56

(1) Copping by biological method in present study.
(2) Davis & Newland by microbiological method on same materials.
(3) Barton-Wright [1942] by microbiological method.
(4) Andrews *et al.* [1942] by microbiological method.
(5) Hodson & Norris [1939] by fluorimetric method.
(6) Conner & Straub [1941*b*] by fluorimetric method.

colorimetric method by means of which he found values of 3·0 and 7·6 μg. per g. for white flour and whole wheat. These values are rather higher than those obtained by the biological method but of the same order. Recently, Conger & Elvehjem [1941] have described a biological method for estimating vitamin B_6 by the growth of rats which is essentially the same as that used in the present study. While these workers did not include cereals among the materials which they examined, so that no comparative values exist, it is worthy of note that the rates of weight increase of their control animals receiving vitamin B_6 hydrochloride in daily doses of 2, 5 and 10 μg. were, respectively, 9, 14 and 19 g. weekly, and in the present tests for rats receiving 2·5 and 5 μg. they were 10 and 14 g. weekly, respectively. This would seem to indicate that the methods in these two laboratories were directly comparable and that biological testing of vitamin B_6 can now be said to be on a sound basis.

The results of the tests for filtrate factors in flours and offals are important in so far as they give a comparison of the value of the different milling fractions and indicate the distribution of the filtrate factors through the grain. This appears to follow that of vitamin B_6 fairly closely, a considerable amount of both vitamins being found in the white flour, with a marked increase in the transition from 73 to 85% extraction and a less marked increase in the further advance to 100% extraction. A great increase in the amount in flour of 85% as compared with that in 73% extraction also held for riboflavin, but the amounts present in the flours and offals were relatively lower than the amounts of vitamin B_6 and filtrate factors. The germ and the weatings or middlings fraction of the grain proved very good sources of all three vitamins, and it is the inclusion of these fractions in the 85% extraction national wheatmeal which raises its nutritive value with regard to all members of the vitamin B_2 complex so greatly above that of white flour.

SUMMARY

1. Estimations have been made by biological methods, with the weight increase of young rats as criterion, of the riboflavin, vitamin B_6, and filtrate factors present in white flour, national wheatmeal and wholemeal, and in wheat germ, bran and middlings or weatings.

2. All the flours were poor sources of riboflavin, but there was a threefold increase with the rise of extraction from 73% for white flour to 85% for national wheatmeal. Wholemeal was only slightly superior to national wheatmeal in its riboflavin content. Wheat germ contained per g. more than three times as much riboflavin as wholemeal, while bran and weatings were about twice as rich in this factor as wholemeal.

3. The differences in vitamin B_6 content in white flour, national wheatmeal and wholemeal were less marked than those in riboflavin content, indicating a more even distribution of vitamin B_6 throughout the grain. Wheat germ and weatings contained per g. about three times as much vitamin B_6 as wholemeal.

4. The amounts of filtrate factors in the flours and offals showed a similar distribution to the amounts of vitamin B_6.

5. The results are summarized in Tables 2–4, and in Table 5 riboflavin values for wheat products obtained by several workers are collected for comparison.

My thanks are due to the Medical Research Council for a part-time personal grant, to Miss A. S. Cole for her assistance during the latter part of this investigation, and to the Lister Institute Division of Nutrition, now kindly housed by Sir Charles Martin, for hospitality. Most particularly I have to thank the Research Association of British Flour Millers for their assistance in supplying the materials for study. I wish also to thank Glaxo Laboratories Ltd. for liver extracts, Roche Products Ltd. for supplies of pure riboflavin and vitamin B_1 and Merck and Co. Inc. of Rahway, N.J., for vitamin B_6 hydrochloride.

Throughout the investigations Dr H. Chick and Sq.-Ldr. T. F. Macrae have been most helpful with their advice and criticism. I acknowledge gratefully the co-operation of Dr Davis and Mr Newland, of the National Institute for Research in Dairying, Shinfield, and of Dr Fraenkel, of the Imperial College of Science and Technology, in making tests of the riboflavin content of my materials by other methods.

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The Intravenous Glucose Tolerance Equation

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The intravenous glucose-tolerance test in which the blood-sugar is measured at intervals for an hour or two following a single intravenous injection of glucose has received steady but not intensive attention during the last 20 years. Recent important contributions include those of Crawford [1938], Ross [1938], Lozner, Peters, Taylor & Winkler [1941], Hamilton & Stein [1942], McKean, Myers & von der Heide [1935] and Tunbridge & Allibone [1940]. The two last-named papers contain reviews of the literature up to 1935 and 1938 respectively.

The lack of agreement among the various workers regarding procedure is remarkable. This applies not only to the dose of glucose given, but also to the assessment of the results. As the latter involves interpretation of the curve obtained for each test when the blood-sugar is plotted against the time after injection, a study of such curves seems most desirable. This paper concerns curves obtained in a series of glucose tolerance tests performed in this Hospital in 1940 in collaboration with Dr Derek Russell Davis; I undertook recently an examination of them. The following data are important for an appreciation of what follows; fuller practical details are given later in the paper.

In all, 35 tests were carried out on 13 adult psychotic patients. In 26 tests the dose of glucose was 15 g. per sq. m. body surface, in 8 it was 0·5 g. per kg. body weight, and in 1 test it was 27·6 g. [cf. Tunbridge & Allibone, 1940]. The glucose was injected in 33⅓% solution in water over a period of 2 min., and the time at which the blood specimens were taken was measured from the mid-time of the injection. 14 specimens were taken at intervals from 5 to 90 min. (both inclusive) after, and 4 specimens before the administration of glucose. The blood-sugar (in mg./100 ml.) will be denoted by y, the time (in min.) by t, and the graph obtained by plotting y against t will be called 'the curve'. Two of the curves obtained are shown in Figs. 1 and 3 (points represented by circles).

AN EQUATION FOR THE INTRAVENOUS GLUCOSE TOLERANCE CURVE

A simple relationship which might hold approximately is that the rate of fall of blood-sugar at any instant after the injection of glucose is proportional to the value of the blood-sugar at that time, i.e.

$$\frac{dy}{dt} = -Ky, \qquad \text{(i)}$$

where K is a constant. This gives on integration

$$\log y = -Kt + \text{const.} \qquad \text{(ii)}$$

Such a relationship has been found to hold approximately for the disappearance of foreign sugars from the blood [Fishberg, 1930], and also for the disappearance of calcium ion from the blood following

the intravenous injection of calcium salts [Greville, 1931], although in the latter case y represents not the blood-calcium but its excess above the initial value. If equation (ii) holds for the intravenous glucose-tolerance curve, then when $\log y$ is plotted against t a straight line should be obtained. In their recent paper Hamilton & Stein [1942] have shown that this is true to a limited degree. The points in the middle part of the plot of $\log y$ against t (corresponding to blood-sugars between 100 and 200–250) fall approximately on a straight line; at both ends the points usually deviate, in the direction of increasing y. Now a positive value of y which will be denoted by y_0. The equation which applies is therefore

$$y = -\frac{1}{k}\frac{dy}{dt} + y_0,$$

so that on rearrangement we have instead of equation (i)

$$\frac{dy}{dt} = -k\,(y - y_0), \qquad \text{(iii)}$$

where k, the slope of the line, is given in Fig. 1 by LY/Yy_0. Thus at any instant, the rate

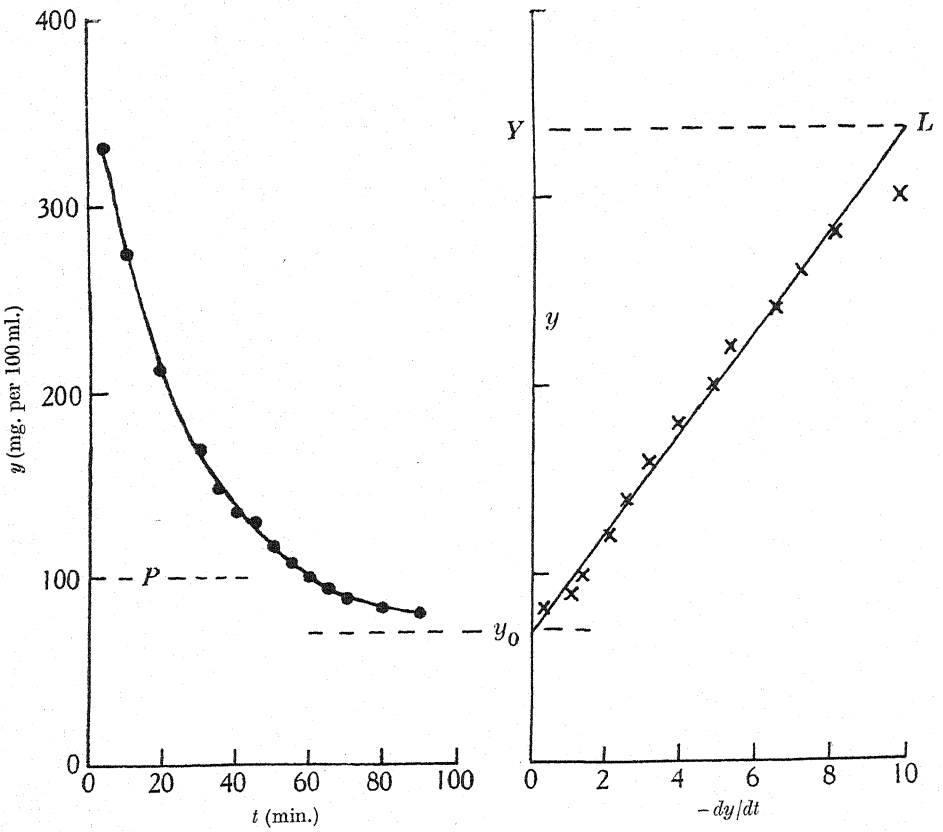

Fig. 1. Intravenous glucose tolerance test. Circles (•), blood-sugar (y) plotted against time (t) (curve fitted by hand). Crosses (×), dy/dt (slope of y, t curve) against y. P = pre-injection blood-sugar. For other symbols see text.

if equation (ii) holds, equation (i) should also apply, in which case dy/dt plotted against y should give a straight line which passes through the origin. In Fig. 1, dy/dt, which is the slope of the y, t curve, has been measured for several values of y, and then plotted against y (crosses). The points fall approximately on a straight line, which, however, does not pass through the origin, but intersects the y-axis at

of fall of blood-sugar is proportional not to the blood-sugar, but rather to the excess of the blood-sugar above some value y_0, which is a constant for the particular test. In that of Fig. 1 y_0 equals 70.

Integrating (iii) we have

$$\log (y - y_0) = C - kt, \qquad \text{(iv)}$$

where C is a constant, and expressing equation (iv) in the exponential form,

$$y = e^{C-kt} + y_0. \qquad (v)$$

Equation (iv) shows that when log $(y-y_0)$ is plotted against t a straight line should result. This actually occurs, as will be seen from the graph marked '$y_0=70$' in Fig. 2. This was obtained by plotting log $(y-y_0)$ against t, using the data of Fig. 1 and the value of y_0 (70) derived above. The points fall close to a straight line over the whole range of the measured blood-sugar values. A still closer fit is shown in Fig. 3 (graph III) for another test ($y_0=52$). With all 35 tests of this series it was found that when log $(y-y_0)$ is plotted against t the points lie about a straight line for the whole, or nearly the whole, of the range 5–90 min. We may therefore state that, for the series of tests under consideration, equation (iv) represents the data of any test with considerable accuracy; and equation (v) is an intravenous glucose-tolerance equation in the exponential form.

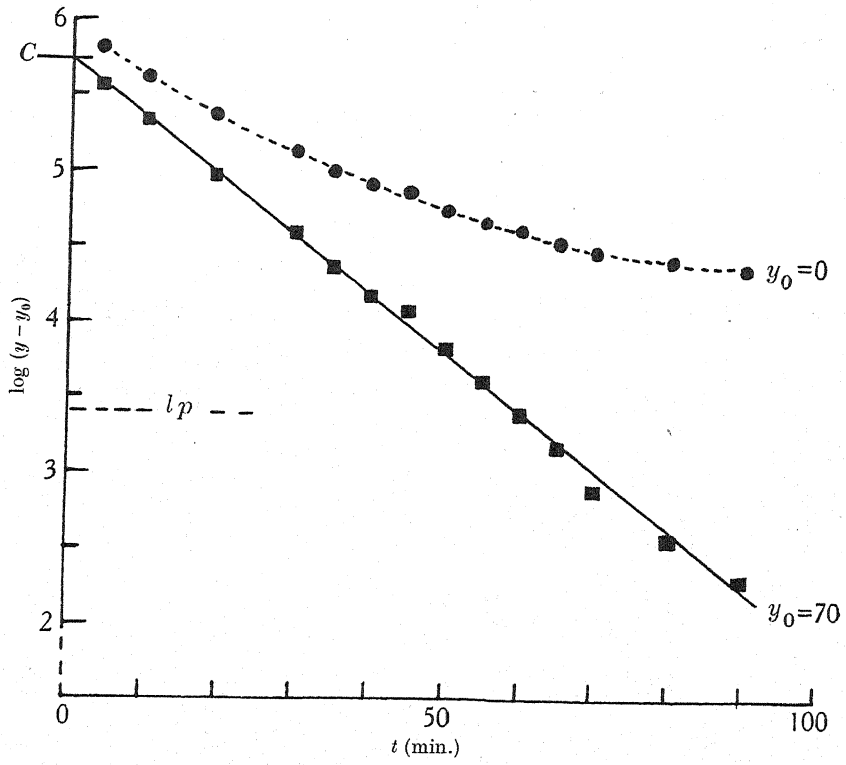

Fig. 2. Same test as Fig. 1. '$y_0=70$', log $(y-70)$ against t (straight line fitted using constants of regression equation). '$y_0=0$', log y against t. *lp*: value of log $(y-70)$ corresponding to pre-injection blood-sugar.

Estimation of the constants of the equation

Method 1: graphical. This, the method of choice, follows the principles already outlined. y is first plotted against t, and the smooth curve drawn which seems best to fit the points. Then the slope of the curve is measured graphically for several values of y, usually chosen at intervals of 20 mg./100 ml., more frequently where the curve has greatest curvature. It is best to take the mean of two measurements of the slope for each value of y. The values thus obtained for the slope (dy/dt) are plotted against the corresponding values of y, as in Fig. 1. The straight line which best seems to fit the points is drawn; it intersects the y-axis at y_0. y_0 is read off, log $(y-y_0)$ calculated for each blood specimen, and plotted against t, as in Fig. 2. The points are fitted by eye with a straight line; k is given by the slope of the line, and C by the point at which it intersects the log $(y-y_0)$ axis.

Method 2: trial and error. Log $(y-y_0)$ is plotted against t several times, using different arbitrary values of y_0 each time, until that value of y_0 is found which brings the points most nearly in a straight line. k and C are then measured from this line as in method 1. Method 2 is somewhat laborious and not very sound, but is occasionally useful.

Method 3: calculation. Let (y_1, t_1), (y_2, t_2), (y_3, t_3) be three points on the curve separated by equal time

intervals, so that $t_2-t_1=t_3-t_2$. Then it can be shown that if equation (iv) holds,

$$y_0=(y_2^2-y_1y_3)/(2y_2-y_1-y_3). \qquad \text{(vi)}$$

I am indebted to Dr Tudor Jones for pointing out this relationship. The method is to read off from the

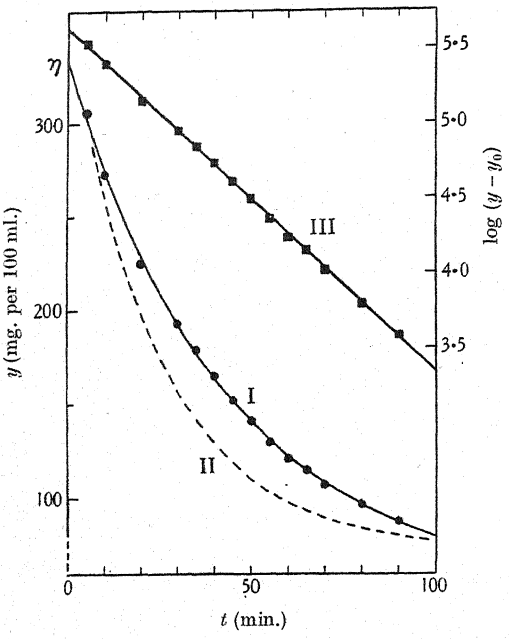

Fig. 3. A different test from that of Fig. 1. Circles (●), observed values of y plotted against t. I (solid curve) represents the equation $y=e^{C-kt}+y_0$, where $C=5{\cdot}638$, $k=0{\cdot}02310$, $y_0=52$. II (broken curve) represents the same equation with k increased to 0·04 and y_0 increased to 72. III, log $(y-52)$ against t (straight line fitted using constants of regression equation).

smooth y, t curve the values of y at (say) 10, 35 and 60 min. y_0 is then calculated by equation (vi). At the same time k can be calculated by the equation

$$k=\frac{1}{t_2-t_1}\log\frac{y_1-y_2}{y_2-y_3}. \qquad \text{(vii)}$$

Equations (vi) and (vii) have, in general, not yielded very accurate results, but once again the method has proved useful on occasion.

Notes on method 1

(*a*) The logarithms in the above equations and figures are of course natural logarithms, to the base e. One can if necessary plot logarithms to the base 10, and multiply the values of C and k found from the graph by $\log_e 10=2{\cdot}30$.

(*b*) Fitting a straight line to the $(dy/dt, y)$ points is sometimes a little difficult. There is often a systematic departure from the straight line with the first one or two points where y is large, which is much more marked in this graph than in the log $(y-y_0)$, t plot. These deviating points are best neglected and the line fitted to the remainder. Again, small deflexions of the y, t curve may produce considerable changes in the slope, and the dy/dt, y plot may be slightly sigmoid as in Fig. 1.

In order to get some idea of the error involved in the drawing of the y, t curve and the measurement of slope, the y, t curve was drawn twice for each of 34 tests. Each curve was used to construct a dy/dt, y curve, so that for each test there were two evaluations of y_0. Statistical examination showed that in 95 tests out of 100 the difference between the two evaluations would not be expected to exceed 15 (standard error = 4·89).* Experience showed that when y_0 was used to construct a log $(y-y_0)$, t curve and hence to estimate k, alteration of y_0 by 15 caused an error of about 13·5 % in the estimate of k. This is not great, considering that the range of k in the series is from 0·016 to 0·039.

Another check on the suitability of the graphical method arises from the circumstance that the dy/dt, y graph should have the same slope k as the log $(y-y_0)$, t graph. For each of the 34 tests, the mean of the two estimates of y_0, derived from two drawings of the y, t curve as above, was used to construct the log $(y-y_0)$, t graph and hence obtain an estimate of k. The slopes of the two dy/dt, y graphs also gave two estimates of k, of which the mean was taken. The arithmetic mean of k from the whole series of log $(y-y_0)$, t graphs was 0·0268, and from the whole series of dy/dt, y graphs 0·0270. In any individual case a series of approximations should strictly be made until the two values of k agree satisfactorily, but the first approximation was generally found to give good enough agreement for all practical purposes, and the closeness of the means of the two sets supports the decision to neglect the small differences.

Goodness of fit: statistical examination

In an attempt to assess the goodness of fit, the linear regression equation relating Z to t was calculated, namely,

$$Z=C-kt,$$

where $Z=\log(y-y_0)$, and C and k are constants, $-k$ being the regression coefficient of Z on t. r^2, the square of the correlation coefficient between Z and t, was then found. r^2 is a measure of the fraction of the variability of Z which is attributable to the linear relationship.

The regression equations have been computed for six tests only, as it would be laborious and of no great worth to calculate them for all; the constants can be

* It should be recognized that the standard error used here is not a measure of the accuracy with which y_0 is determined, but a measure of the consistency of results obtained by the graphical method.

estimated satisfactorily by the graphical method. In order to obtain representative figures the tests were divided into three groups, 'best', 'medium' and 'worst', having regard to the amount of scattering of the points round the y, t curve. They were thus probably assessed according to the amount of some of the tests there seem to be systematic deviations which need consideration.

It is difficult to fix any standard with which to decide whether a point deviates from the line. It is best to examine the graphs with a sense of proportion. I have judged as fairly as I could for each

Table 1. *Intravenous glucose-tolerance test protocols, showing simultaneous values of blood-sugar y (mg./100 ml.) and time t (min.), together with the values of the constants y_0, C and k in the equation log $(y-y_0)=C-kt$ calculated for each test*

No.	1		2		3		4		5		6	
Sex, age	F, 47		F, 46		F, 36		M, 38		M, 54		M, 44	
Diagnosis*	*Mel.*		*C.Ps.*		*Mel.*		*Mel.*		*Par.*		*Mel.*	
Weight (kg.)	44·5		52·6		47·2		51·3		64·9		49·4	
Surface area (m.²)	1·43		1·53		1·45		1·59		1·66		1·61	
Glucose injected (g.)	21·3		26·3		21·6		24·0		25·0		24·0	
y_0	63		73		50		78		61		64	
C	5·68		5·64		5·55		5·24		5·56		5·38	
10^4k	304		385		239		208		259		289	
	t	y	t	y	t	y	t	y	t	y	t	y
Initial value	—	94	—	85	—	85	—	87	—	99	—	88
	5	323	5	309	5	314	5	259	5	299	5	256
	10	264	10	262	10	261	10	231	10	257	10	223
	21	220	20	201	20	205	20	197	20	209	20	185
	26	195	30	167	30	178	30	181	30	182	30	151
	37	162	35	149	35	164	35	170	35	168	35	144
	41	142	40	137	40	148	40	159	40	156	40	136
	46	144	45	123	45	140	45	152	45	154	45	125
	51	130	50	110	50	127	50	145	50	129	50	116
	56	115	55	107	60	112	55	139	55	120	55	104
	60	106	60	98	65	104	60	132	60	115	60	97
	65	103	65	97	70	100	65	128	65	110	65	96
	70	98	71	90	80	87	70	120	70	104	70	92
	80	89	80	84	90	82	80	115	80	93	80	86
	85	84	90	84	—	—	90	108	90	88	90	80

* *C.Ps.* confusional psychosis; *Mel.* melancholia; *Par.* paraphrenia.

experimental error. The two most representative tests of each group were then selected. The results are given in Table 2. The calculations were done for the whole of the range 5–90 min., except that in no. 1 the last specimen was at 85 min. Test no. 7 is that represented in Fig. 3; r^2 is here as high as 0·9989.

Table 2. *Constants of the regression equation $Z=C-kt$, where $Z=\log(y-y_0)$*

Group	No. of test	y_0	C	10^4k	r^2
Best	7	52	5·638	231·0	0·9989
	4	78	5·245	207·6	0·9973
Medium	1	63	5·690	306·9	0·9957
	2	73	5·637	384·9	0·9906
Worst	8	63	5·510	335·8	0·9594
	9	55	5·280	217·8	0·9592

Goodness of fit: range

Above we have assessed the goodness of fit of the straight line given by equation (iv) to the data over the whole experimental range. In Figs. 2 and 3 (III) the points lie close to the line throughout, but in test, the first and last points which lie close to the line.

In 10 tests out of 31 the 5 min. point was judged to deviate, in each case above the line, so that the blood-sugar was falling more rapidly than equation (iii) allows. The 10 min. point deviated in only 2 out of 35 tests. The blood-sugar corresponding to the first point lying near the line was noted for each of 35 tests; the average was 275 mg./100 ml. (range 187–332).

In 13 tests the 90 min. point deviated above or below the line; in 19 it did not. The average of the 35 blood-sugar values corresponding to the last point near the line was 90 (range 75–137). In 16 tests out of 30, equation (iv) continued to be obeyed after the blood-sugar had fallen below the pre-injection value, in one case as far as 24 mg./100 ml. below.

Goodness of fit: exponential curve

A demonstration of the goodness of fit consists in reconstruction of the y, t curve. Thus, the constants of equation (iv) for test no. 7 (Fig. 3) having been accurately determined (Table 2), they are inserted

in equation (v) so that y can be calculated for any t. For the blood specimens we have

t	5	10	20	30	35	40	45
y calc.	302·5	275	229	193	177	163·5	152
y obs.	306	273	225	193	179	165	152
t	50	55	60	65	70	80	90
y calc.	140·5	131	122	114·5	108	96·5	87
y obs.	141	130	121	115	107	96	88

In Fig. 3 the solid curve (I) represents equation (v) with the appropriate values of the constants, i.e.

$$y = e^{5\cdot638 - 0\cdot02310t} + 52,$$

and the circles represent the experimental values of y.

These results speak for themselves.

The constants

y_0, which might be called the 'limiting value of y', is the level which the curve would approach asymptotically were equation (v) to be obeyed when t becomes large. What happens when y actually approaches y_0 cannot be stated; none of the tests were continued after 90 min., and all the 90 min. values of y were greater than y_0. In Fig. 1 the curve seems to be approaching y_0 with precision. In two curves y reached a minimum (approx. 75 and 68) and then rose again. The values of y_0 in these tests were low (25 and 32 respectively), so that y rose long before y_0 was reached, otherwise hypoglycaemic signs might have appeared. y_0 does not appear to be correlated with the pre-injection blood-sugar, but in all tests it was smaller than the latter. y_0 ranged from 25 to 91.

k, as is seen from equation (iii), gives the rate of fall of blood-sugar per unit excess of blood-sugar above the limiting value y_0. The unit is min.$^{-1}$. Throughout the tests there was a slight tendency for k to increase with y_0. k ranged from 0·0176 to 0·0386.

C. If equation (iv) were to hold for values of t approaching zero, then C would be the value of $\log(y - y_0)$ when $t = 0$. If the corresponding value of $(y - y_0)$ be denoted by B, and that of y by η, then $\eta = B + y_0$ and equation (iv) becomes

$$y - y_0 = Be^{-kt}. \qquad \text{(viii)}$$

C ranged from 5·12 to 5·82, B from 167 to 337, and η from 234 to 369.

Non-psychotic subjects

As many psychotics show abnormal oral glucose-tolerance curves, the above findings should be controlled by observations on non-psychotic subjects. I have taken data from the literature, thus covering different experimental conditions, and found C, k and y_0 for a considerable number of tolerance tests. The results with a few of these are shown in Table 3. Except with nos. 2, 8 and 9, it was necessary to measure the published graphs to obtain y and t.* Some of the values of y_0 and k in these tests fall outside the range found with the psychotics. The columns headed 'Range of t' and 'Range of y_0' show the range over which equation (iv) was judged to apply. This was wide, especially with nos. 6 and 9.

EXPERIMENTAL DETAILS

In view of Himsworth's well-known work, the patients were kept on a constant carbohydrate intake of 282–299 g. daily. Absence of infection was checked by determinations of the erythrocyte sedimentation rate. The tolerance tests were performed in the morning, after a fast of about 15 hr. The glucose was injected with the aid of a three-way tap fitted to the syringe nozzle, and 0·1 ml. blood specimens were taken from the ear-lobe [see Harrison, 1937].

Table 3. *Constants of the intravenous tolerance equation in non-psychotic subjects*

Reference	Glucose injected	y_0	10^4k	C	Range of t	Range of y	Description
(1)	?	96	1068	5·58	2–30	296–106	Normal curve
(2)	0·5 g./kg.	65	334	5·61	2–75	298–88	? children
(3)	0·5 g./kg.	150	686	5·17	3–30	270–171	Child, 11 yr., rheumatism
(4)	30 g.	85	453	5·48	3½–51½	291–110	Normal man, aged 32 yr.
(5)	15 g.	80	555	5·06	1½–44½	226–94	Same subject as no. 4
(6)	27·6 g.	62	375	5·56	2½–64½	305–85	Normal medical student aged 21 yr.
(7)	27·6 g.	105	263	5·61	10–78	319–142	Fractured femur, man, aged 68 yr.
(8)	25 g.	59	289	5·42	5–120	254–67	Normal male, aged 52 yr.
(9)	20 g.	56	334	5·55	4–112	280–62	Heart disease, female aged 15 yr.

(1) Ross [1938], Fig. 17. (2) Crawford [1938], Table 1, case 1, test *a*. (3) Ibid. Fig. 4. (4) Tunbridge & Allibone [1940], Fig. 4, curve I. (5) Ibid. Fig. 4, curve II. (6) Ibid. Fig. 10, curve II. (7) Ibid. Fig. 11, curve I. (8) Lozner *et al.* [1941], Table 1, no. 5. (9) Jörgensen [1926–7), no. A$_{XIV}$.

Blood-sugar was determined by the cerimetric direct titration method of Miller & Van Slyke [1936], which gives values very close to the 'true' blood-

* Since many workers present their results in the form of diagrams only, it is difficult to use their work for comparison. Protocols of six tests in the present series are given in Table 1, in case any reader would care to use them for checking the assertions made here.

sugar, and about 7 mg./100 ml. lower than the Hagedorn-Jensen values. Methyl violet 5 *B* was found to be an excellent indicator [cf. Vanossi & Ferramola, 1936].

DISCUSSION

Blood-sugar dynamics. The remarkable thing which has emerged is that the rate of disappearance of glucose from the blood, although presumably the resultant of a number of simultaneous processes, conforms with considerable accuracy to a simple equation.

In the first few minutes the blood-sugar is clearly higher than is to be expected from equation (v). In several tests even the 5 min. value of y is greater than η, the value of y at $t=0$ given by equation (v). On the other hand, Table 3 shows that the $1\frac{1}{2}$ and 2 min. points can on occasion lie approximately on the calculated curve. There is, however, an extremely rapid disappearance of glucose from the blood during a short period after the injection.

Thus, with test no. 7 (Fig. 3), 21·4 g. of glucose were injected into a 44·0 kg. woman. The average blood volume of women, according to Harington, Pochin & Squire [1940], is 72·3 ml./kg., so here it would be 3·18 l. The blood-sugar should therefore rise immediately by 673 mg./100 ml.; hence from the pre-injection level of 89 it should rise to 762. For this test, however, η was calculated to be 333, and the 5 min. value of y was 306. Even if the blood volume were as high as 5 l., y would be expected to rise to 517. Several authors have made a somewhat similar calculation (cf. also Greville [1931] on the injection of calcium salts).

A working hypothesis, unsupported by direct evidence, for the events following the injection of glucose is as follows. The blood-sugar reaches a maximum quickly,* and then falls extremely rapidly, partly due to osmotic entry of water into the blood, but mostly to rapid diffusion of glucose from the blood into the tissue fluids. The glucose is rapidly spread over the 'active volume of the tissue fluids' [Burn & Dale, 1924; Orr-Ewing, 1931]. After a time the blood-glucose will come into dynamic equilibrium with the tissue fluid-glucose. It is now suggested that when this occurs, the rate of fall of the blood-sugar conforms to equation (iii). The disappearance of glucose from the blood at this stage mirrors, as it were, the net metabolic removal of glucose in the tissues. Finally, at a low blood-sugar level, processes tending to raise the blood-sugar achieve predominance, the curve rises, and equation (v) no longer holds.

* Although McKean *et al.* [1935] and Fairley [1936] found that the maximum occurs some minutes after the injection, probably owing to slow mixing of the glucose solution with the blood, the curves of Tunbridge & Allibone [1940] and Crawford [1938] show that the maximum usually occurs before the end of the first minute.

Equation (iii) shows that the rate of fall of blood-sugar bears a straight-line relationship to the blood-sugar level. In experiments in which the rate of glucose utilization was estimated at various maintained blood-sugar levels, it has been found that the former bears an approximate straight-line relationship to the latter, both in the eviscerated, hepatectomized dog [Soskin & Levine, 1937] and in the perfused hind-limb of the cat [Lundsgaard, Nielsen & Ørskov, 1938]. Thus the present findings with a rapidly-falling blood-sugar, and the interpretation of these findings, are in conformity with the observations with steady glycaemic levels.

Properties of the curve. It is seen from equation (v) that when y_0 is altered the curve shifts bodily upwards or downwards. k largely determines the shape of the curve. Thus in Fig. 3, the broken curve (II) was obtained by increasing k to 0·04 from 0·0231, the value applying to the solid curve; increasing k has caused the curve to bend more sharply. (y_0 was also increased, to facilitate comparison by raising the curve.) Similarly, decreasing k would straighten the curve somewhat. When C (or B) is altered, the values of $(y-y_0)$ are all changed in the same ratio (equation viii).

Assessment of tolerance tests. The methods which have been used to characterize the results of intravenous glucose-tolerance tests should now be examined in the light of the findings of this paper.

The time taken for the blood-sugar to return to the pre-injection level [Tunbridge & Allibone, 1940], or to 100 mg./100 ml. [Crawford, 1938], or the value of the blood-sugar at a certain time after the injection [Lozner *et al.* 1941] are criteria which do not seem at first to give any information about the shape of the curve, which depends on k. Nevertheless, any or all of these may possibly be correlated with k; indeed, in the series of tests on psychotics there appears to be a rough relationship between k and the time taken for the blood-sugar to return to 95, one tending to increase as the other decreases.

Ross [1938] has used as a criterion the area under the curve bounded by certain arbitrary limits. Despite the criticisms of this by Tunbridge & Allibone [1940], one should not dismiss it before satisfying oneself that the area is not correlated with any of the characteristics of the curve.

The procedure of Hamilton & Stein [1942] needs more detailed comment. These workers plotted log y against t, and found that equation (ii) is approximately obeyed in the middle part of the graph. In Fig. 2, log y has been plotted against t using the data of Fig. 1. The graph is labelled '$y_0=0$', since equation (iv) becomes identical with equation (ii) when y_0 is put equal to zero. It is clearly curved, in contrast to the plot for the same data of log $(y-y_0)$ against t when $y_0=70$. It is true that in several of the other tests the graph of log y against t is much straighter

in the middle part than that in Fig. 2; but an examination of all the tests leaves no doubt that equation (iv) gives a very much better representation of the data than does equation (ii). The constant K of equation (ii) multiplied by 100 was proposed as a measure of the test. Since it then gives the rate of fall of blood-sugar at any instant as a percentage of the blood-sugar at that instant (equation (i)), it would be very convenient. Unfortunately, as we have seen, equation (ii) does not represent the data well, and it is not easy to determine K by drawing a straight line through points lying on a curve.

When the test is used in the diagnosis of disease, any criterion may be taken which proves adequate in practice. For example, Lozner *et al.* [1941] showed that the blood-sugar 2 hr. after the injection of 25 g. of glucose is a reliable guide for distinguishing between benign glycosuria and diabetes mellitus. They established a mean normal value for the 2 hr. blood-sugar, and also found the standard deviation. Any value differing from the mean by more than three times the standard deviation was considered abnormal. Of course in any test the blood-sugar may have passed through a minimum during the first 2 hr., but this does not matter: the criterion is justified by experience.

We have seen that the curve is characterized by three constants, k, y_0 and C. It is unfortunate from the practical point of view that one is not enough. However, the intravenous glucose tolerance test is regarded as a 'delicate instrument' [Ross, 1938], which 'deserves a somewhat more accurate method of measuring' than is obtained by noting the time at which the blood-sugar returns to normal levels [Hamilton & Stein, 1942]. These views suggest that it may prove necessary in the future to characterize experimental curves by the constants of the tolerance equation, though experience may establish a connexion between any one or any combination of the constants and any of the factors, normal and pathological, on which the outcome of the test depends.

SUMMARY

1. In the intravenous glucose-tolerance test, following a single injection of glucose, the value of the blood-sugar (y) is related to the time (t) after the injection by the equation

$$\log (y - y_0) = C - kt,$$

where y_0, C and k are constants, the values of which vary from test to test.

2. This relationship is obeyed with considerable accuracy over all, or nearly all, of the time period 5–90 min. after the injection. It was found to apply in a series of tests on psychotics, and also in numerous tests on normal subjects taken from the literature.

3. The intravenous glucose-tolerance curve thus approximates to an exponential curve of the form

$$y = Be^{-kt} + y_0,$$

where $\log B = C$; and at any instant the rate of fall of blood-sugar is proportional to the excess of the blood-sugar above the 'limiting value' y_0.

4. Methods for determining the constants of the equation are described, and an attempt is made to estimate the goodness of fit.

5. The assessment of the results of intravenous glucose-tolerance tests is discussed in the light of these findings.

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The Assay of Riboflavin in Cereals and other Products

1. MICROBIOLOGICAL ASSAY

2. FLUOROMETRIC ASSAY

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1. MICROBIOLOGICAL ASSAY

The fluorometric methods of estimating riboflavin developed by Hodson & Norris [1939], Najjar [1941] and others have so far proved to be unsatisfactory in our hands when applied to cereals, for reasons referred to in Part 2. The microbiological method introduced by Snell & Strong [1939], however, with suitable modifications has been found to be satisfactory and expeditious.

EXPERIMENTAL

Organism. The organism used was *Lactobacillus helveticus* from Snell & Strong's original strain no. 7469 obtained from the American Type Culture Collection, Georgetown University, Washington, D.C.* A number of different strains of *L. helveticus* were tested for a quantitative response to riboflavin, but with the exception of a strain from Dr J. G. Davies of the National Institute for Research in Dairying, Reading, and which originated in U.S.A., none gave a quantitative response to riboflavin. The majority grew equally well in the absence of the vitamin, while others gave an 'all-or-none' reaction.

The technique employed for the growth of the organism and for the assay of riboflavin was similar to that employed by Snell & Strong with certain modifications which are described below.

Stock cultures of the organism were carried on a yeast-water glucose agar. The cultures were kept in a refrigerator at a temperature of approximately 4° and removed at monthly intervals.

Basal medium. The composition of the riboflavin-free basal medium for the determination of the vitamin was the same as that recommended by Snell & Strong, with, however, the further addition of asparagine, xylose, pantothenic and nicotinic acids. The composition of 250 ml. of the final medium, sufficient for 50 assay tubes was as follows:

50 ml. photolysed NaOH-treated peptone solution; 50 ml. cystine solution; 12·5 ml. of a 1 % asparagine solution; 5·0 g. glucose; 0·25 g. xylose; 2·5 ml. solution A;* 2·5 ml. solution B; 5 ml. yeast supplement and nicotinic and pantothenic acids so that every ml. of the medium contains 0·05 μg. of these two substances.

Media and extracts were always made with glass-distilled water; this precaution is essential.

Preparation of inoculum. The procedure described by Snell & Strong for the preparation of fresh batches of inoculum for assay purposes was not found to be satisfactory, as there was frequently heavy growth in the riboflavin-free blanks. On this account a fresh inoculum was always prepared from the stock agar culture for each set of determinations.

The presence of xylose in the medium has a two-fold action. In the first place the results between replicates were found to be more concordant whether direct titration with 0·1 *N* NaOH or a nephelometric method were employed for the actual determination, and in the second place, in the case of nephelometric determinations there was a larger 'spread' between concentration levels.

The fermentations were carried out in ordinary chemical or bacteriological test-tubes (18 × 150 mm.) and the technique described by Snell & Strong was strictly followed.

Two methods have been used to determine quantitatively the response of the organism to added riboflavin. In the first the turbidity produced by the growth of the bacteria is measured. This procedure is difficult with highly coloured extracts and special precautions have to be taken. In the second method the acid produced during growth is determined by direct titration with 0·1 *N* NaOH, using bromothymol blue as indicator.

If a nephelometric method be employed, then it is necessary on statistical grounds to have five repli-

* We are indebted to Dr J. C. Drummond for obtaining this culture from Washington.

* Solution A is composed of 25 g. K_2HPO_4 and 25 g. KH_2PO_4 dissolved in 250 ml. distilled water. Solution B contains 10 g. $MgSO_4.7H_2O$, 0·5 g. NaCl, 0·5 g. $FeSO_4.7H_2O$. 0·5 g. $MnSO_4.4H_2O$ dissolved in 250 ml. distilled water. Solution B tends to deposit a dark coloured precipitate upon long standing, so that the solution should be well shaken before use.

cates for each level of concentration. Snell & Strong consider that two are sufficient. This number, however, is too small and the results cannot be relied upon. Moreover, for the unknown solutions whose riboflavin content is to be determined there must also be at least three 'blanks' for each concentration level. These 'blanks' are inoculated in the same way as the other tubes and placed in a refrigerator so that bacterial growth is prevented. The readings for the blanks are subtracted from the values obtained for the tubes in which growth has taken place.

The estimation of acid formed during fermentation, however, was found to be the most convenient method. It is simpler and less cumbersome than a nephelometric method and fewer tubes are required for an assay. Here again, Snell & Strong recommend only two tubes for each concentration level. It is, however, from the practical standpoint, much more satisfactory to use three, and this number was always employed in the present investigation.

For the actual titration a comparator was used. The procedure adopted was the same as that described by Cole [1928] for the estimation of amino-acids. Instead of the contents of the tube being washed into a conical flask and then titrated to *p*H 6·8 using a colour-comparison flask to obtain the necessary end-point as Snell & Strong recommend, the titration was carried out in the fermentation tube itself. A great saving of time is effected by this procedure, and the end-point is clearer and more easily determined than with a colour-comparison flask.

With this method the titration is carried out as follows:

A buffer solution of *p*H 6·8 is prepared by mixing 50 ml. of a 0·2*M* solution of KH_2PO_4 with the equivalent of 23·65 ml. of 0·2*N* NaOH, and diluting to 200 ml. 20 ml. of this solution is then pipetted into a test-tube (18 × 150 mm.) and 20 drops of bromothymol blue indicator added. 20 ml. of distilled water is pipetted into another tube. Two of the tubes of which the acid content is to be determined are now taken and to one is added 10 drops of indicator and the tubes are then arranged in the comparator as shown in the following diagram:

A B

C D

Tube A contains distilled water and tube B buffer solution + indicator. Tubes C and D contain the assay solutions and to C indicator is added. No indicator is added to D.

Tube C is now titrated with 0·1*N* NaOH, and for every ml. or part of a ml. of standard alkali added an extra drop of indicator is also added, while to tube D the same amount of standard alkali is run in from the burette. The titration is completed when the colours seen through C and D exactly match.

Preparation of extracts. It has been shown by Scott, Randall & Hessel [1941] that starch has a marked effect in stimulating the growth of *L. helveticus*, and as cereals contain a large amount of starch, this must first be destroyed in the extract before the estimation is carried out. Scott *et al.* used taka-diastase for this purpose. In the present investigation, however, taka-diastase was found to be unsatisfactory. The various samples of the enzyme that were tested showed a marked stimulatory action over other hydrolysing agents, such as ptyalin, H_2SO_4 and HCl. The method of extraction that was eventually adopted was to hydrolyse suitable weighed samples of material with 0·25*N* HCl for 15 min. at 15 lb. pressure in an autoclave. For cooked wheaten products, such as bread, the time of hydrolysis must be increased to 30 min. Nevertheless, hydrolysis with ptyalin in place of taka-diastase or refluxing for 1 hr. with 0·25*N* H_2SO_4 [Hodson & Norris, 1939] or with 0·25*N* HCl were all found to give the same results as autoclaving for 15 min. Autoclaving of the material was adopted simply because it was found to be more convenient. After hydrolysis the *p*H of the extract was adjusted to 6·8 with NaOH and made up to the requisite volume. For the great majority of cereals it was found that a 5 g. sample of the finely ground material hydrolysed with 50 ml. of 0·25*N* HCl and made up to 500 ml. was a convenient quantity for assay. For substances with a low riboflavin content, such as patent flours, 10 g. samples were used. When a high riboflavin content was suspected, e.g. germ, 1 g. samples were used. A convenient rule to follow is to have the extract of such a strength that it contains a total of approximately 10–12 μg. of riboflavin. For each assay three different levels of concentration must be used. The results should not differ among themselves by more than 10 %, and the mean of the values is taken as the riboflavin content. In the present investigation the levels of concentration taken were, 2, 3 and 4 ml. of the extract.

The results from the different methods of extraction that were employed are given in Table 1.

Table 1

Sample	Riboflavin content μg./g.
National Straight Run flour (75 % extraction)	
1. Hydrolysis with ptyalin	0·75
2. Refluxed with 0·25*N* H_2SO_4 for 1 hr.	0·75
3. Refluxed with 0·25*N* HCl for 1 hr.	0·80
4. Autoclaved at 15 lb. pressure for 15 min. with 0·25*N* HCl	0·80
5. Hydrolysed with taka-diastase	1·15

Fats, like starch, exert a stimulating effect upon the growth of *L. helveticus*, and substances with a high fat content, such as soybean, maize, germ and milk, must first be defatted before an

extract is made. Cereals with a high fat content are best defatted by extraction in a Soxhlet extractor with light petroleum. Milk should be separated on a centrifuge and the serum shaken up in a separating funnel with ether. Light petroleum is not a suitable fat solvent with milk, as the mixture tends to form an emulsion.

The stimulating effects of fats on *L. helveticus* are shown by the results in Table 2.

Table 2

Sample	Riboflavin content μg./g.
Milk	
1. Raw	1·95
2. Separated	1·66
3. Separated and extracted with petrol ether	1·66
4. Separated and extracted with ether	1·45

EXPERIMENTAL RESULTS

The results of a number of determinations on different varieties of wheat, various fractions of the wheat grain, wheaten flours and other cereals are given in Table 3; figures are based on the normal water contents of the products.

Table 3

Sample	Riboflavin content μg./g.
1. Wheat, English	1·9–2·65
2. Wheat, Manitoba	2·7–2·9
3. Wheat, Australian	2·4–2·65
4. Wheat, Russian	3·7
5. Wheat, Testa	3·9–5·8
6. Wheat, Aleurone layer	6·3–7·55
7. Wheat, Germ. Commercial sample 1	11·35
8. Wheat, Germ. Commercial sample 2	12·85
9. Wheat, Germ. Commercial sample 3	9·7
10. Flour, A roll	0·4–0·5
11. Flour, C roll	0·5–0·6
12. Flour, National Straight Run (75% extraction)	0·75–1·25
13. Flour, National Wheatmeal (85% extraction)	1·7–2·1
14. Barley	2·3
15. Rye	2·65
16. Maize	2·3–2·7
17. Soybean	3·0–4·9

The riboflavin content of samples of baker's yeast and brewer's yeast was also determined.

	Riboflavin content μg./g.
Baker's yeast	23·0
Brewer's yeast	66·0

The possible destruction of riboflavin by baking was investigated. A control loaf and a loaf prepared from the same flour to which 2 μg./g. of synthetic riboflavin had been added were assayed. The crumb was air dried in the dark and in both cases had a moisture content of 6·7 % (Table 4).

Table 4

	Riboflavin content μg./g.
1. Control loaf	2·85
2. Loaf + 2 μg./g. added riboflavin	4·80
3. Recovered riboflavin	1·95

It will be seen from the results in Table 4 that there is no significant destruction of riboflavin in the baking process.

A survey of the riboflavin content of different milling fractions of an English wheat which was specially milled by our colleague Mr A. G. Simpson gave the results in Table 5.

Table 5

Sample; English wheat (dry milled, moisture content 14·0 %)

	Milling fraction %	Riboflavin content μg./g.	Contribution of riboflavin to Straight Run flour %	Contribution of riboflavin in whole wheat %
Break and scratch flours	23·4	0·9	18·8	11·1
Semolina reduction flour	7·2	0·5	3·5	2·1
Middlings (dunst reduction flour 1)	43·6	0·6	23·2	13·7
Middlings (dunst reduction flour 2)	5·4	4·4	22·7	13·4
Middlings (dunst reduction offals, retreated flour)	1·9	6·2	10·7	6·3
Bran and scratch offals, retreated flour	4·1	5·8	21·5	12·7
Straight Run flour (Calculated value)	85·6	1·5 (1·32)	100·4	59·3
Bran	6·0	6·9	—	18·0
Pollards	5·0	6·3	—	13·6
Fine offals	3·4	6·3	—	9·4
Whole wheat (deter.)	100·0	2·4	—	100·3
Whole wheat (calc.)	—	2·3	—	—

This wheat was milled in the laboratory by a special technique designed to give the maximum amount of white flour. It differs from that which can be normally manufactured in commercial practice.

2. FLUOROMETRIC ASSAY

Two major difficulties have been encountered in this estimation when carried out by any of the previously published fluorimetric methods: (1) the presence of colloidal matter and/or fine suspended particles in the final riboflavin solution as prepared for visual or photoelectric assessment of its fluorescence; this

material cannot be removed by centrifuging or normal filtration and its effect in absorbing and scattering the blue exciting light can easily be so great as to mask completely any fluorescence; (2) the initial low concentration of riboflavin in wheat and the majority of the products thereof which causes the fluorescence of final solutions derived from such materials to be too small to be measurable with any degree of accuracy.

The first of these difficulties was overcome by recourse to the procedure of Najjar [1941] in which the riboflavin is extracted by a non-aqueous medium; by this means all suspended material is left in the aqueous layer.

The second difficulty is of a more serious nature—adsorption of the riboflavin from a fairly large quantity of a wheat extract, followed by elution by means of a small volume of liquid was considered to be most worthy of trial as also by this means various interfering fluorescent materials present in the extract might be eliminated.

Freshly precipitated lead sulphide (as recommended by Najjar for urines) was found to be unsatisfactory for wheat or flour extracts; as also was English fuller's earth, even of grades specially selected for adsorption purposes. Through the kindness of Dr B. S. Platt opportunity was afforded of trying out an American fuller's earth of the 'white' variety ('Superfiltrol') and this has proved satisfactory.

The method which follows has been elaborated around the 'Spekker' fluorimeter but may be adapted readily to other types of fluorimeters.

EXPERIMENTAL

20 g. of finely ground wheat, flour or dried bread (or quantities of milling offals containing approx. 20–50 μg. of riboflavin) are taken in a 250 ml. flask and are suspended with shaking in 100 ml. of 0·25N H_2SO_4. Two further flasks are made up in the same way but with the addition of 10 and 20 μg. respectively of riboflavin, conveniently added as 1 and 2 ml. respectively of a 10 μg./ml. standard solution. The flasks are plugged with cotton wool and are autoclaved for 15 min. at 15 lb. pressure. (In order to ensure complete hydrolysis of pre-cooked wheaten products such as bread, it is advisable to prolong autoclaving to 30 min.—see Part 1.)

After cooling, the digests are adjusted to approx. pH 5 by the dropwise addition of 30 % NaOH, using brom-cresol green as an external indicator. They are adjusted to a convenient volume (125 ml.) and are filtered through a no. 5 Whatman paper using suction. The filtrate should at this stage be crystal clear.

Three 50 ml. conical centrifuge tubes are prepared, each containing 0·5 g. ($\pm$ 5 mg.) of 'Superfiltrol' and 45 ml. aliquots of each digest are added to them. The fuller's earth is then maintained in suspension for 5 min. by brisk mechanical stirring, a fine glass rod bent at the tip being convenient for the purpose. After centrifuging, the supernatant liquor is carefully poured off and discarded and approx. 20 ml. of distilled water is added to each tube. After agitation for 2 min. in order to wash thoroughly the earth the tubes are again centrifuged and the washing water decanted off. The tubes are then allowed to drain in an inverted position for a few minutes, after which all traces of water adhering to the inside of the tube are wiped away as far as possible with a clean glass cloth.

Elution of adsorbed riboflavin is carried out by the addition of 8 ml. of a reagent with the following composition: 9 vol. of water, 5 vol. of pyridine (redistilled) and 2 vol. of glacial acetic acid. Mechanical stirring is again employed for 5 min., after which the tubes are centrifuged at slow speed for 5 min.

The eluate from each tube is decanted as completely as possible into suitable glass-stoppered flasks or cylinders, with a total capacity of not less than 33–34 ml. (25 ml. measuring cylinders are usually of this total capacity and are convenient for the purpose.)

To each vessel is added 3 drops of 4 % $KMnO_4$ solution; it is mixed and allowed to stand for at least 1 min. to oxidize interfering substances. Two drops of 10 vol. H_2O_2 will then be found sufficient to decolorize the solutions.

5 g. of anhydrous Na_2SO_4 and 20 ml. of n-butyl alcohol are added to each vessel, the vessels are immersed in a water bath to raise the temperature of their contents to about 35–40°, and are then shaken vigorously for 2 min. After cooling, the whole of the supernatant layers, together with as little of the aqueous layers as possible, are decanted into centrifuge tubes and are spun at slow speed for 5–10 min. It will then be found that sufficient of the butyl-alcohol pyridine layer, now perfectly clear, may be decanted to fill the fluorimeter cell. Measurement of fluorescence is made using blue activating light (Wratten no. 47 filter) and an orange filter (Chances) in front of the photocell. A convenient standard green fluorescing solution is a 1 μg./ml. solution of sodium fluorescein in weak alkali.

Blank determinations on each sample are carried out after destruction of the riboflavin contained therein by exposure to the unscreened radiation of a mercury-vapour arc or to direct sunlight for 1–2 hr. according to the method of Najjar.

INTERPRETATION OF RESULTS

Within a limited range of concentrations, Beers' law is obeyed by solutions of riboflavin. As the drum of the 'Spekker' fluorimeter is calibrated logarithmically, a linear ratio is obtained between drum reading and concentration over this range.

Under the conditions outlined above it is found that this linearity of ratio holds, and therefore a straight line may be drawn relating the fluorescences of the three flour extracts to their added riboflavin contents. The 'blanks' of these three extracts will be identical within the limits of experimental error, and this 'blank' figure is used on the extrapolation of the graph from which may be read the amount of riboflavin present in the total quantity of wheat, flour, bread, etc., which was taken in the first place.

In Table 6 are given some results which have been obtained by the method. They include a number of samples which have been assayed by the microbiological method and which appear in Tables 3 and 4.

Table 6

Sample	Riboflavin content μg./g.
1. Wheats, English (1 in Table 3)	1·7–2·3
2. Wheat, mixed grist	2·4
3. Germ (8 in Table 3)	14·5
4. Bran (6·8 μg./g. by microbiological method)	7·4
5. National Wheatmeal sample (*a*)	1·5
6. National Wheatmeal sample (*b*)	2·0
7. Control loaf (1 in Table 4)	2·6
8. Control loaf + 2 μg./g. added riboflavin)	4·4

Duplicate estimations by the method on different days have given results differing on all occasions by less than ± 10 %.

DISCUSSION

The fluorometric method as outlined above is essentially a modification of that of Najjar. No assumption is made that adsorption or elution of riboflavin is complete—the method depends upon keeping volumes and quantities of reagents, etc., constant as between fortified and unfortified extracts, and the use of a standard solution of riboflavin eliminates calculations on such points, which would otherwise be necessary. This method was chosen as being simpler and more expeditious than adopting the directly quantitative system of multiple adsorptions and elutions, though the occasional adoption of such a procedure provides a useful check.

It is found that whereas for one product of fairly constant composition (e.g. National Wheatmeal flour) the gradient of the fluorescence curve is reasonably constant (and consequently it may be found possible, with the sacrifice of a little accuracy, to make riboflavin additions to occasional samples only), this gradient varies markedly from one wheat or milling product to another, and needs to be separately determined on each sample.

For this reason, and despite all efforts which have been made to simplify the fluorometric technique with a view to making it as expeditious as possible, it must be admitted that it is in our experience a very much more laborious and time-consuming method than the microbiological.

The values found by both methods in the present investigation for the riboflavin content of different cereals show that there are considerably greater amounts of this vitamin present in these products (about 10 times) than is indicated by the literature [Fixsen & Roscoe, *Nutr. Abstr. Rev.* 1939–40]. Recently, Andrews, Boyd & Terry [1942] have published figures for the riboflavin content of wheat and wheaten products, as well as other cereals, obtained by the Snell & Strong microbiological method. With the exception of patent flour, the values they obtained are all much lower (approx. half) than those now reported. One explanation for these low figures is that their extracts were too concentrated (5–10 g. made up to 100 ml.). In our experience there is always a falling off in estimated riboflavin if the extract be too concentrated. There is also the further possibility that the *p*H of the extracts was not correctly adjusted. If the *p*H of the extracts be lower than 6·6 the riboflavin values will be found to be too low. Kemmerer [1942] has reported the results of 18 collaborators in a comparison between the fluorometric and microbiological method for the assay of riboflavin in alfalfa meal, wheat and flour. Good agreement was obtained between the various collaborators for alfalfa meal by either method, but extremely wide variations were obtained for wheat and flour, whichever method was used. For example, the values for wheat by the fluorometric method varied between 0·53 and 2·6 μg./g. and with the microbiological method the variations were 0·78–2·7 μg./g., while for flour both fluorometric and microbiological methods gave a range of 0·15–1·5 μg./g. Such extremely wide variations in results between different workers are difficult to understand. In our experience with the microbiological method fluctuations of the order reported by Kemmerer were never encountered.

That the values reported here are reliable is shown firstly by the fact that the determined and calculated values obtained from the different milling samples (see Table 5 above) agree within experimental error. Secondly, the values obtained for the same samples of cow's milk obtained by one of us (E. C. B.-W.) working with the microbiological method and by Dr S. K. Kon of the National Institute for Research in Dairying working independently at Reading with the fluorometric method, agree within experimental error (see Table 7).

Table 7

Sample	Riboflavin content (μg./g.)	
Cow's milk	Microbiological method	Fluorometric method
1	1·6	1·5
2	1·4	1·32
3	1·5	1·4

SUMMARY

The microbiological method of Snell & Strong and the fluorometric method of Najjar for the assay of riboflavin have been improved and modified with particular reference to cereals and cereal products. Agreement between the results obtained by the two methods is good, and figures are presented covering a wide range of such products.

The microbiological method is preferred as it is more rapid than the fluorometric, and requires less material and no specialized apparatus.

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The Effect of Salts on the Formation of Protein Complexes during Heat Denaturation

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The separation of floccules from solutions of proteins denatured by heat is the final and most obvious stage in a process of aggregation. The factors controlling flocculation have been studied in detail by many workers, but little attention has been given to their effects on earlier stages of aggregation. If the heating is insufficient, or other conditions are unsuitable to cause flocculation, stable solutions of heat-changed proteins are obtained. When solutions containing more than one protein are heated in such conditions, aggregation may take place between particles of different proteins, when stable solutions of protein complexes are produced. Such complexes may have properties different from those of either the unheated mixture of proteins or the individual proteins heated separately. For example, such complexes are formed when serum albumin and globulin are heated together; these have been detected by electrophoresis [Van der Scheer, Wyckoff & Clarke, 1941], as well as by changes in the ease of sedimentation during centrifugation, and by changes in precipitability with salts [Kleczkowski, 1941*a*].

Antibodies and protein-antigens can combine to form complexes with other proteins simultaneously undergoing denaturation by heat. Van der Scheer *et al.* [1941] and Krejci, de Spain Smith & Dietz [1941] showed that when antisera are heated, antibodies are found in products with changed electrophoretic mobility, and Kleczkowski [1941*b*] and Bawden & Kleczkowski [1941, 1942] have described changes in the serological behaviour of both antibodies and protein-antigens after heating with unspecific serum proteins. These changes are most striking when 'O'-type antigens, giving granular precipitation with antisera, or their antibodies, are heated in the presence of serum albumin. The complexes formed by such antigens are no longer precipitated by antibodies, although they still combine with them to inhibit the precipitation of any unheated antigens added subsequently. Similarly, the complexes formed by antibodies still combine with the antigens but are unable to precipitate them.

In this paper the effects of salts on the formation of such complexes are described and compared with their effects on the separation of floccules.

MATERIAL AND METHODS

The proteins used for studying the changes in precipitability produced by the formation of complexes were the 'soluble euglobulin' and albumin fractions from rabbit serum. The proteins used as antigens were tomato bushy-stunt virus, and the soluble whole globulin and albumin fractions from human serum. These were all prepared by the methods previously described [Kleczkowski, 1941 *a*, *b*; Bawden & Kleczkowski, 1941].

The antisera used were against tomato bushy-stunt virus, against the whole globulin of human serum and against human serum albumin. They

were produced from rabbits by intravenous injections.

Dialysis was carried out in cellophane sacs for 3 days against distilled water changed three times a day. To prevent any bacterial decomposition of serum proteins, a little thymol was put into the sacs.

The protein solutions were heated in thin-walled tubes immersed in a water-bath.

The precipitability of heated protein solutions was measured by finding the concentration of $MgSO_4$ necessary to cause precipitation. When a precipitate was formed, it was allowed to settle by leaving the fluid undisturbed for 3 days. Then the supernatant was decanted, and the precipitate washed three times by resuspending in $MgSO_4$ solutions of an appropriate concentration and centrifuging. The N content was determined by a micro-Kjeldahl method, and the results expressed in terms of protein by multiplying by 6·25.

RESULTS

The effect of NaCl on the formation of complexes between soluble euglobulin and albumin fractions of rabbit serum

Table 1 shows the results of heating the soluble euglobulin and albumin fractions from rabbit serum separately and together, both in the presence and absence of NaCl. All the solutions were heated for 10 min. at 80° at *p*H 7·0. It will be seen that when heated in the presence of salt a product was formed in the protein mixture, whose precipitability with $MgSO_4$ differed widely from either protein fraction heated separately. The amount of the product was larger than the total euglobulin in the mixture, so that albumin as well as globulin must have contributed to its formation.

Table 1. *The effect of NaCl on interaction between globulin and albumin fractions of rabbit serum during heating*

The amount of proteins in 4 ml. of heated solutions (mg.)		The amount of precipitate formed during heating (mg.)	The amount of precipitate formed with the following concentrations of $MgSO_4$ (% saturation) (mg.)				
Euglobulin	Albumin		30	50	70	80	100
		Heating with 1 % NaCl					
0	15	0	0	0	0	0	0
5	0	4	.	.	.	.	.
5	15	0	0	0	0	8	.
		Heating in salt-free solutions					
0	15	0	0	0	0	0	0
5	0	0	3·2	.	.	.	.
5	15	0	0	4	.	.	.
5	0 mixed after	0	3·2	.	.	.	.
0	15 heating						

*p*H of the dialysed solutions was adjusted to 7·0 by adding 0·1 *N* NaOH. The solutions were heated for 10 min. at 80°.

Serological precipitin tests were made by adding 1 ml. of antigen solutions to 1 ml. of antiserum solutions, so that the range of antigen/antibody ratios, indicated in the tables, was covered. The tubes were immediately placed in a water-bath at 50° with the fluid columns half immersed, so that convection currents ensured complete mixing. The appearance of floccules was taken as a positive result. The ability of treated antigens to combine with antibody without causing precipitation was measured by their ability to inhibit precipitation. After 3 hr. incubation at 50°, appropriate amounts of unchanged antigen were added to the tubes in which there were no floccules. The absence of precipitation after this addition of precipitable antigen was taken as evidence of inhibition by the treated antigen. Similarly, the ability of treated antibody to combine with antigen without causing precipitation was tested by adding untreated antiserum to the tubes in which there was no precipitation.

When the salt-free mixture of the proteins was heated, a product was also formed whose precipitability differed from that of either protein heated separately. This effect was not due to mixing the proteins, for when the two were heated separately and then mixed, their precipitabilities were the same as when tested separately. Thus during heating together both in the presence and absence of salt, there is an interaction between the two proteins leading to formation of a product with new properties. The amount of the product, however, is much greater when the heating is carried out in the presence of salt, and its precipitability then also differs more widely from that of the euglobulin heated alone. These results suggest that in the presence of salt euglobulin combines with considerable quantities of albumin, whereas in the absence of salt it combines with only small quantities to give a product whose properties differ only slightly from those of the euglobulin heated alone.

The effect of salts on the formation of serologically non-precipitating complexes between human and rabbit serum albumins during heating

The euglobulin and albumin fractions of rabbit serum differ so widely in their precipitability with $MgSO_4$ and $(NH_4)_2SO_4$, both before and after heating, that complex formation between them can be readily detected by changes in the precipitability with these salts. This method, however, can obviously not be applied to proteins with similar precipitabilities, such as the albumin fractions from two different animal sera. The formation of complexes often influences the serological reactions of protein-antigens, so that serological methods can be applied for their detection in systems where precipitation with salts is unsuitable. In the tests described below the inability of complexes to precipitate with the antisera, and also their ability to inhibit specifically the precipitation of unchanged

Table 2. *The effect of salts on the formation of non-precipitating complexes between human serum albumin and rabbit serum albumin*

Concentration of salt (normality) in heated solutions	Appearance of the solutions after heating	Precipitin test Human albumin mg. 0·5	0·25	0·12	0·06	0·03	0·015	0·0075
Unheated human albumin		−	+	+	+	+	+	+
Heating at *p*H 7·0								
R Salt-free	clear	+	+	+	+	+	+	+
$Al_2(SO_4)_3$								
R 0·00015	clear	+	+	+	+	+	−	−
R 0·0003	clear	+	+	i	i	0	0	0
R 0·0006	sl.op.	i	i	i	i	i	0	0
R 0·0012	st.op.	+	+	+	+	0	0	0
R 0·0025	pptt.	.	.	.	.	.	.	.
H 0·0006	clear	−	+	+	+	+	+	+
$MgSO_4$								
R 0·0006	clear	+	+	+	+	−	−	−
R 0·0012	clear	+	+	i	i	0	0	0
R 0·0025	sl.op.	i	i	i	i	i	i	0
R 0·005	st.op.	i	i	i	i	i	0	0
R 0·01	st.op.	+	+	+	0	0	0	0
R 0·015	pptt.	.	.	.	.	.	.	.
H 0·0025	opal.	+	+	+	+	+	+	−
Heating at *p*H 3·2								
R Salt-free	clear	+	+	+	+	+	+	+
$BaCl_2$								
R 0·05	clear	+	+	+	+	+	+	+
R 0·2	opal.	i	i	i	i	0	0	0
H 0·2	clear	+	+	+	+	+	+	+

Concentration of salt (normality) in heated solutions	Appearance of the solutions after heating	Precipitin test Human albumin mg. 0·5	0·25	0·12	0·06	0·03	0·015	0·0075
$BaCl_2$								
R 0·0006	clear	+	+	+	i	0	0	0
R 0·0012	clear	+	+	i	i	i	0	0
R 0·0025	sl.op.	i	i	i	i	i	i	0
R 0·005	st.op.	−	+	+	+	+	+	+
R 0·01	pptt.	.	.	.	.	.	.	.
H 0·0025	sl.op.	+	+	+	+	+	+	−
Na_2SO_4								
R 0·012	clear	+	+	+	i	0	0	0
R 0·05	sl.op.	+	i	i	i	0	0	0
R 0·2	sl.op.	i	i	i	i	0	0	0
H 0·2	sl.op.	+	+	+	+	+	+	+
NaCl								
R 0·006	clear	+	+	+	0	0	0	0
R 0·012	clear	+	+	i	i	0	0	0
R 0·025	sl.op.	+	i	i	i	i	0	0
R 0·05	sl.op.	i	i	i	i	i	i	0
R 0·2	sl.op.	i	i	i	i	i	i	0
R 0·3	opal.	i	i	i	i	+	+	+
R 0·6	st.op.	−	−	+	+	+	+	+
H 0·2	sl.op.	+	+	+	+	+	+	+
Na_2SO_4								
R 0·003	clear	+	+	+	+	+	+	+
R 0·006	sl.op.	+	+	i	i	0	0	0
R 0·012	opal.	i	i	i	i	i	0	0
R 0·025	pptt.	.	.	.	.	.	.	.
H 0·012	sl.op.	+	+	+	+	+	+	+

Precipitin test. 1 ml. of an antiserum to human albumin at a dilution 1/40 was added to 1 ml. of antigen solutions at various concentrations. + indicates precipitation and − no precipitation if test for inhibition was not made. Inhibition was tested by addition of 0·1 ml. of 0·03% solution of unheated human albumin to the mixtures where there was no precipitation. i indicates inhibition, 0 indicates no inhibition.

The solutions were heated for 10 min. at 83°.

R, heated solution contained 0·05% of human and 0·5% of rabbit albumin.

H, heated solution contained 0·05% of human albumin.

sl.op. slight opalescence; opal. moderate opalescence; st.op. strong opalescence; pptt. precipitation.

antigens, have been used as a test for their formation [cf. Bawden & Kleczkowski, 1941].

The formation of complexes between albumin from human serum and albumin from rabbit serum was studied, using an antiserum to human albumin. Dialysed solutions of the two albumins were adjusted to *p*H 7·0 and *p*H 3·2 by adding 0·1 *N* NaOH or HCl respectively. They were then heated separately and in mixtures containing ten times as much rabbit albumin as human albumin. They were heated for 10 min. at 83° salt-free and in the presence of various salts at different concentrations. After cooling, the solutions at *p*H 3·2 were adjusted to *p*H 7·0 by adding 0·1 *N* NaOH. All the heated solutions were diluted in physiological saline and tested serologically for their ability to give precipitation or inhibition with human albumin antiserum. The results are given in Table 2.

Human albumin heated separately at either *p*H 7·0 or 3·2, salt-free, or in the presence of all the tested salts, precipitated with the antiserum to human albumin. This specific precipitation was not affected if rabbit albumin, either unheated or heated separately, was mixed with the heated human albumin. The salt-free solutions of the mixtures of human and rabbit albumins, heated at either *p*H, also precipitated with the antiserum. Heating these mixtures in the presence of all the salts, on the other hand, produced non-precipitating complexes that inhibited the precipitation of human albumin added subsequently.

For each salt four zones of concentration can be distinguished, in each of which the effect of heating the protein mixture is different. At low concentrations no serologically demonstrable complex is formed and heated solutions are precipitated by the antiserum. In the next zone a serologically non-precipitating complex is formed. Heating in the third zone gives material that is precipitated specifically by the antiserum. This material flocculates with antiserum more quickly than the unheated human albumin and forms larger floccules. In the zone of highest salt concentration a precipitate of denatured protein separates during heating.

Hardy [1900] showed that the efficiency of salts in flocculating colloidal solutions (including proteins) is determined by the valency of the ion with the charge opposite to that of the colloidal particles. The efficiency is expressed as the reciprocal of the lowest concentration, in terms of normality, necessary to cause flocculation. Ions of higher valency are much more efficient than those of lower valency. Chick & Martin [1912] found that Hardy's law also applies to the efficiency of salts in flocculating protein solutions previously denatured by heat in the absence of salts. The results given in Table 2 show that this law also applies to the formation of complexes between two different proteins. At *p*H 7·0, i.e. on the alkaline side of the isoelectric point of heated proteins, when the protein particles are negatively charged, the efficiency of salts in causing the complex formation is determined by the valency of the cations. The lowest concentration of $Al_2(SO_4)_3$ causing the complex formation was approximately one-tenth of that of $MgSO_4$ or $BaCl_2$, and this was approximately one-tenth of that of Na_2SO_4 or NaCl. At *p*H 3·2, i.e. on the acid side of the isoelectric point of the heated proteins, when the protein particles are positively charged, the efficiency of salts in causing the complex formation depends on the valency of the anions. The lowest concentration of Na_2SO_4 causing the complex formation was approximately one-tenth of that of $BaCl_2$.

The fact that the efficiency of salts in promoting the complex formation is determined by the valency of the ion charged oppositely to the protein particles, strongly suggests that the action of salts is electrostatic. They can be pictured as acting by discharging the protein particles, thus facilitating their combination with each other, as when they cause flocculation of colloidal suspensions. The formation of protein complexes, however, is not altogether comparable with flocculation. Flocculation is caused whether salts are present during the heating or are added to the protein solutions after heating. The formation of protein complexes, however, takes place to any appreciable extent only when the mixtures are heated in the presence of salt. If salt-free mixtures of human and rabbit serum-albumin fractions are heated and salt added after cooling, no material that inhibits serological precipitation can be detected, and the heated mixture precipitates with the antiserum to human albumin in exactly the same way as human albumin heated alone.

The effect of salt on the formation of a serologically non-precipitating complex between tomato bushy-stunt virus and rabbit serum albumin during heating

Table 3 shows the influence of NaCl on the formation of serologically non-precipitating complex between tomato bushy-stunt virus and rabbit-serum albumin. In salt-free solution no demonstrable amount of the complex was formed, and the heated virus-albumin mixture behaved serologically like a solution of the virus heated alone. When heated in the presence of salt, a non-precipitating complex was formed, and the lowest concentration of NaCl causing the complex formation was approximately equal to the lowest NaCl concentration causing formation of the complex between human- and rabbit-serum albumins (cf. Table 2).

With this system also, the addition of salt to the virus-albumin mixture previously heated salt-free did not cause the formation of any demonstrable quantities of a non-precipitating complex.

Table 3. *The effect of NaCl on the formation of non-precipitating complexes between tomato bushy-stunt virus and rabbit serum albumin*

Concentration of NaCl (normality) in heated solutions	Appearance of the solutions after heating	Precipitin test Dilutions of the antiserum 1/20	1/40	1/80	1/160	1/320	1/640
Unheated bushy-stunt virus		+	+	+	+	+	−
Heated virus-albumin mixture:							
0·012	clear	+	+	+	+	−	−
0·025	sl.op.	0	0	0	i	i	−
0·05	sl.op.	0	i	i	i	i	−
0·1	opal.	i	i	i	i	i	−
Salt-free	clear	+	+	+	+	+	+
The virus heated alone:							
0·1	st.op.	+	+	+	+	+	+

Precipitin test. 1 ml. of every antigen preparation diluted 1/10 in physiological saline was mixed with 1 ml. of an antiserum to bushy-stunt virus at varying dilutions.
Inhibition was tested by adding 0·1 ml. of 0·05% solution of the unheated virus.
The solutions were heated for 10 min. at 83° at *p*H 7·0.
Virus-albumin mixture contained 0·05% of bushy-stunt virus and 0·5% of rabbit serum albumin.
The virus alone was used as 0·05% solution.
The symbols as in Table 2.

The effect of salt on the formation of a serologically non-precipitating complex between soluble human serum globulin and rabbit serum albumin during heating

Table 4 shows that when the water-soluble fraction of human-serum globulin was heated with rabbit-serum albumin, rather different results were obtained. In the presence of salt the system resembled the other two described above; a serologically non-precipitating complex was formed which inhibited the precipitation of the unheated globulin added later. The mixture heated in the absence of salt, however, also failed to precipitate with the antiserum, although it did not give the phenomenon of inhibition. When the globulin was heated alone, it still precipitated specifically with the antiserum whether rabbit albumin was later added or not. Thus the change in behaviour of the salt-free mixture was a result of an interaction between the two proteins during heating.

It has been shown above, by measuring changes in the precipitability with $MgSO_4$, that there was some combination between rabbit euglobulin and albumin when a mixture was heated salt-free, although much less than when heated in the presence of NaCl. The results in Table 4 suggest that a complex was also formed between human globulin and rabbit albumin when heated salt-free. It appears that the complex was formed in an amount sufficient to destroy serological precipitability but not sufficient to produce the inhibition phenomenon, which was well pronounced when NaCl was present during heating and the complex formation occurred to a greater extent.

Table 4. *The effect of NaCl on the formation of non-precipitating complexes between soluble human serum globulin and rabbit serum albumin*

Treatment of human globulin	Appearance of the solutions after heating	Precipitin test Human globulin mg. 0·5	0·25	0·12	0·06	0·03	0·015	0·0075
Unheated		+	+	+	+	+	+	+
Heated with albumin in 1% NaCl	st.op.	i	i	i	i	0	0	0
Heated with albumin salt-free	clear	0	0	0	0	0	0	0
Heated alone in 1% NaCl	pptt.	.	.	.	.	.	.	.
Heated alone salt-free	st.op.	+	+	+	+	−	−	−

Precipitin test. 1 ml. of an antiserum to human globulin at a dilution 1/10 was added to 1 ml. of antigen solutions at various concentrations.
Inhibition was tested by adding 0·1 ml. of 0·06% solution of unheated soluble human globulin.
The solutions were heated for 10 min. at 83° at *p*H 7·0.
In all heated solutions concentration of human globulin was 0·05% and that of rabbit albumin 0·5%.
The symbols as in Table 2.

The effect of salt on the formation of non-precipitating complexes between antibodies and unspecific serum proteins during heating

Antibodies can undergo initial stages of heat denaturation without losing their ability to combine with their antigens, and, like protein-antigens, when heated in the presence of other proteins undergoing denaturation simultaneously, they can combine with them to form complexes [Kleczkowski, 1941*b*]. If combined with sufficient albumin, they do not precipitate their antigens, but, as they still combine with them, they interfere with the action of precipitating antibodies. The degree of this interference depends on the nature of antigen, being large with 'O'-type antigens and small with 'H'-type antigens. Heating whole antisera can lead to the formation of such complexes produced by heat-changed antibody particles combining with serologically unspecific proteins present in the sera.

To test the effect of salt on the formation of such antibody complexes, dialysed antisera to human-serum albumin and to tomato bushy-stunt virus were used. The precipitate of insoluble globulin formed during dialysis was filtered off and the filtrate was used as test solution. This involved loss of antibodies, but sufficient remained in solution for the test. The results of heating the dialysed antisera with and without addition of NaCl are given in Table 5.

DISCUSSION

All the antigens used in this work belong to the group of proteins which, when heated in the presence of salt with serologically unspecific serum albumin, combine with it to form serologically non-precipitating complexes. In spite of their uniform behaviour in the presence of salt, when heated salt-free they behave differently. Bushy-stunt virus, and human-serum albumin, heated with rabbit-serum albumin in salt-free solutions did not form any demonstrable amounts of non-precipitating complexes; they still precipitated with their antisera exactly as if they had been heated alone. On the other hand, the water-soluble fraction of human-serum globulin heated salt-free in the presence of rabbit-serum albumin formed a non-precipitating complex, although to a smaller extent than in the presence of salt. This complex did not precipitate with the antiserum, but it differed from that formed in the presence of salt as it did not show the presence of inhibition. It is difficult to account for these differences satisfactorily, although various tentative explanations can be offered. Susceptibility to the presence of traces of salts, which might have remained after dialysis, may vary with different proteins, so that complex formation could proceed in globulin-albumin mixtures but not in the two other protein systems. The differences may be apparent rather than real, for only the precipitin test was used to detect the formation of the complexes. Therefore, if the different antigens vary in their sensitivity to the presence of the complexes, they may have been formed equally in all three systems in the absence of salts, but only with globulin may it have been sufficient to prevent precipitation. It is, however, equally possible that the globulin differs from albumin and the virus when heated in the absence of salt, and that the first can combine with rabbit-serum

Table 5. *The effect of NaCl on the formation of non-precipitating complexes between antibodies and unspecific serum proteins*

	The antiserum to human albumin Human albumin (mg.)						The antiserum to bushy-stunt virus Dilutions of the antiserum					
Treatment of the antisera	0·25	0·12	0·06	0·03	0·015	0·0075	1/10	1/20	1/40	1/80	1/160	1/320
Unheated	+	+	+	+	+	+	+	+	+	+	–	–
Heated in 1% NaCl	0	0	i	i	i	i	i	i	i	i	0	0
Heated salt-free	+	+	+	+	+	+	i	i	i	0	0	0

Dialysed antisera were heated for 10 min. at 80° at *p*H 7·0 at a dilution 1/10 in distilled water with and without addition of 1% NaCl.

Precipitin test. The antiserum to human albumin was tested by mixing 1 ml. of the antiserum diluted 1/10 with 1 ml. of solutions of human albumin at various concentrations. The inhibition was tested by adding 0·1 ml. of undiluted dialysed antiserum.

The antiserum to bushy-stunt virus was tested by mixing 1 ml. of 0·005% solution of the virus with 1 ml. of various dilutions of the antiserum. The inhibition was tested by adding 0·1 ml. of the dialysed antiserum diluted 1/2.

The symbols as in Table 2.

Both antisera behaved similarly when heated in the presence of salt, forming non-precipitating and inhibiting complexes. They showed, however, differences after heating salt-free. The antiserum to human albumin still precipitated its antigen, whereas the antiserum to bushy-stunt virus did not precipitate and instead gave inhibition, although the 'inhibition titre' was slightly lower than that given by the same antiserum after heating in the presence of salt.

albumin in these conditions whereas the others cannot.

Whether or not the presence of salt is necessary to start the combination of two proteins, it certainly greatly increases it. As in causing flocculation, the efficiency of different salts in influencing complex formation depends on the valency of the ion with the charge opposite to that of the protein particles. The two processes, however, are not identical. If the protein is denatured by heating in the absence of salts, the subsequent addition of salt causes flocculation. By contrast, salts influence complex formation only if they are present in protein mixtures during heating, for the addition of salt to a protein mixture heated salt-free does not lead to formation of any measurable amount of serologically non-precipitating complexes. This difference could be explained by assuming that individual proteins differ in their rates of aggregation, and that these rates are much greater than rates of denaturation. Then, if salt is present during heating, particles of different proteins would be able to combine with one another as soon as they are denatured, but if salt is added to a mixture of proteins already denatured by heating in salt-free solution, each protein would tend to aggregate separately.

The results with antibodies almost exactly parallel those with antigens. The two antisera used were to bushy-stunt virus and to human-serum albumin. When heated in the presence of salt, these behave similarly, the antibodies forming non-precipitating complexes which inhibit precipitation. In the absence of salts the two antisera behaved differently. The virus antiserum again ceased to precipitate and instead inhibited precipitation, though less strongly than when heated with salt, whereas the antiserum to human albumin still precipitated. This difference at first sight suggests differences in the heat stabilities of the two antisera. A similar difference between the behaviour of antisera to 'O' and 'H'-type antigens is well known and was for long interpreted as evidence that these antigens produced antibodies with different properties. It has now been shown, however, that antibodies to antigens of both types undergo similar changes when heated, and that the difference in their behaviour occurs because the precipitability of the antigens is affected differently by the presence of antibodies changed into non-precipitating complexes [Kleczkowski, 1941*b*; Bawden & Kleczkowski, 1941, 1942]. Similarly, the difference between the behaviour of dialysed antisera to human albumin and to bushy-stunt virus respectively, may be because the two antigens are affected differently by the presence of non-precipitating antibody complexes, which are probably formed to a small extent when both antisera are heated in the absence of salts.

SUMMARY

The formation of complexes between different proteins, undergoing heat denaturation together, is greatly influenced by the presence of salts. In the absence of salts only mixtures containing water-soluble serum globulin formed any detectable amount of complexes.

The efficiency of different salts in promoting the complex formation, expressed as the reciprocal of the lowest effective concentration, follows Hardy's law. Ions of higher valency are much more effective than those of lower valency. On the alkaline side of the isoelectric point of the proteins, the efficiency of salts is determined by the valency of the cations, and on the acid side by the valency of anions.

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The Influence of Halide Concentration on the Metabolism of *Penicillium sclerotiorum* van Beyma

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In a previous communication [Curtin & Reilly, 1940], the isolation and properties of sclerotiorine, a chlorine-containing metabolic product of *Penicillium sclerotiorum* van Beyma, were described. Reference was also made to a second product, a red pigment retained by the mother-liquors of crystallization. Work on the constitution of sclerotiorine was somewhat hampered by the small yield, 2% of

the dry mycelium weight. The possibilities of increasing this figure by alteration of the chloride concentration and by activation with yeast extract [Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941] have now been investigated, the basal medium being that of Czapek-Dox. Simultaneously, the organism has been grown on media devoid of chlorides, viz. Raulin-Thom and Raulin-Dierckx media together with a Czapek-Dox medium compounded without KCl, in the hope of obtaining the red component in a more workable form. The mycelium pigmented in all cases, and the dark red product of light petroleum extraction was submitted to diverse processes to free it from the fats and other products of the extraction. Sublimation in a high vacuum, separation by adsorption on Al_2O_3 and $Ca(OH)_2$, and crystallizations at the interface of two immiscible liquids gave no substance that could be isolated other than as a glaze on glass.

Inoculations of the mould on Czapek-Dox media, containing bromides or iodides in amounts equivalent to the usual KCl concentration, gave no crystalline bromo- or iodo-derivatives. This finds a parallel in the work of Raistrick & Smith [1936]. However, appreciable darkening in the colour of the mycelium and its extract was noted with each change of halide.

Cultural conditions. The culture used throughout was the 6th transfer from that obtained in March 1940 from the Centraalbureau voor Schimmelcultures, Holland. Sucrose was used as the source of carbon, trial cultures proving that this sugar was satisfactory.

The substrate employed was the acid Czapek-Dox medium (sucrose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, modified; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water to 1000 ml.). Four batches, each of 5 l., containing 0·25, 0·5, 0·75 and 1·0 g. KCl/l., respectively as the sole source of chlorine were prepared. The media were distributed in 100 ml. lots in 20 oz. flat bottles, the cotton-wool plugs being paper-capped to prevent dilution of the medium during sterilization by steaming on each of three consecutive days. The bottles were inoculated with a spore suspension from a 14-day-old culture of *P. sclerotiorum* on malt agar, and incubated at 25°. Three bottles were filtered at the pump every 3 days, and the filtrate tested for *p*H, sugar concentration by polarimeter and for residual KCl.

In all inoculations described, the *p*H value reached a minimum on the 9th day and reverted to approximate neutrality on the 24th day, in agreement with the figures already published [Curtin & Reilly, 1940]. The production of sclerotiorine was thus uninfluenced by the acidity of the medium. The polarimeter readings were of particular interest. Due to their similarity, one series will suffice as an illustration (see Table 1).

Table 1

Days of incubation	Polarimeter reading
0	5·7° D
3	2·6° D
6	0·3° L
9	1·0° L
12	0·6° L
15	0·0
18	0·0
21	0·0
24	0·0

The connexion between the minimum *p*H value on the 9th day and the maximum *laevo* reading is obvious. The main interest centres round the fact that more than four-fifths of the sucrose was metabolized in so short a period as 9 days.

Estimation of residual chloride. Direct titration was not possible as the medium reduces both $KMnO_4$ and $AgNO_3$. Oxidation by refluxing with conc. HNO_3 was unsatisfactory. A modified form of the method described by Raistrick & Smith [1936] was finally employed:

50 ml. of the filtered medium were shaken for 10 min. with 2·5 g. of norit charcoal and filtered at the pump without washing. 25 ml. of this filtrate were taken and excess $AgNO_3$ added, together with 5 ml. of 6*N* HNO_3, which had been boiled free from HNO_2. The precipitated AgCl was coagulated by boiling, filtered off and washed with a little dilute HNO_3. On cooling below 25°, the new filtrate was titrated against *N*/100 NH_4CNS, using 1 ml. of standard Volhard indicator.

In actual practice, the medium extracts chlorides from the norit, the KCl equivalent, as determined by a series of blanks, being 0·00095 g./25 ml. of the medium. The residual percentage KCl, at intervals, over a period of 24 days is shown in Table 2.

Table 2

Days of incubation	Initial chloride content of media: 0·25 g. KCl/l.	0·5 g. KCl/l.	0·75 g. KCl/l.	1·0 g. KCl/l.
	Residual chloride content			
	%	%	%	%
0	0·0250	0·0497	0·0747	0·0994
3	0·0172	0·0369	0·0527	0·0762
6	0·0071	0·0274	0·0509	0·0735
9	0·0050	0·0258	0·0499	0·0729
12	0·0070 (?)	0·0250	0·0473	0·0705
15	0·0033	0·0250	0·0470	0·0678
18	0·0027	0·0235	0·0437	0·0661
21	0·0015	0·0233	0·0457 (?)	0·0640
24	0·0009	0·0230	0·0475 (?)	0·0640
Metabolized KCl	0·0241 %	0·0267 %	0·0310 %	0·0354 %

Plotting the residual KCl against time gives four curves of identical shape, which show that the utilization of KCl becomes regular after 9 days. Before this, the halide is rapidly taken up. We have

already stressed the rapidity with which the sugar is utilized during this same period.

The filtered mycelium from 25 bottles (2·5 l.) of each of the four media was pressed, vacuum-dried, and extracted with light petroleum. A mean value of 12·9 g. dry mycelium/l. was taken as the basis of calculation. The extracted sclerotiorine was weighed on recrystallization, the yields of pigment being practically constant, allowing for the necessarily incomplete separation from the solvent (see Table 3, col. 2).

Completion of the chlorine balance sheet was effected by the estimation, as KCl, of the chloride content of the extracted mycelium. The method [Schiff, 1879; 1906] was as follows:

Mycelium (2–3 g.) was weighed into a small platinum crucible and covered with an intimate mixture of 1 part pure Na_2CO_3 and 4 parts pure CaO and inverted into a larger crucible which was then completely filled with the same mixture. The whole was then heated with a blow-pipe so that the outer parts attained a high temperature before the mycelium began to decompose. After heating to redness and cooling, the mixture was treated with distilled water, the solution being acidified with pure HNO_3, an appreciable rise in temperature being avoided. The solution was then adjusted to known volume and the chlorides estimated by the Volhard method already described.

The metabolized KCl and the KCl equivalents of sclerotiorine and the mycelia are given in Table 3. The 5th column, KCl unaccounted for, shows the quantities of KCl retained by the mother-liquors of crystallization, its presence having been duly verified.

Table 3

KCl/l. of medium g.	Yield of sclerotiorine g./l. medium	KCl metabolized as pigment g./100 ml. medium	KCl as mycelium %	KCl unaccounted for %
0·25	0·190	0·0038	0·0127	0·0076
0·50	0·213	0·0042	0·0118	0·0107
0·75	0·189	0·0038	0·0128	0·0144
1·00	0·220	0·0044	0·0136	0·0174

The results so far may be summarized as follows: Yields of pigment and the chlorine content of the mycelium are, within the limits of experimental error, practically constant, while the chlorine content of the mother-liquors are in direct proportion to the KCl concentration of the medium.

Effect of p*H and addition of yeast extract.* A further series of inoculations to test the influence of *p*H and yeast extract on the rate of KCl metabolism was now completed, *p*H, polarimeter and Volhard titrations being performed as already described. For the influence of *p*H, 5 l. of neutral Czapek-Dox medium were made up by K_2HPO_4 instead of KH_2PO_4 as in the acid medium (*p*H 4·2).

Yeast extract was prepared by macerating 500 g. pressed yeast with 1 % soda solution, re-pressing on the Buchner funnel and washing with distilled water. The yeast, suspended in 1 l. of distilled water, was boiled for 1 hr. and allowed to stand overnight, when it was centrifuged and the volume of the clarified extract adjusted to 1000 ml. The extract was then sterilized by steaming on three consecutive days. Two batches of 5 l. standard Czapek-Dox medium were then made up, incorporating 5 and 10 % by volume respectively, of the yeast-water sown and incubated as above. All three media showed the same rapid elimination of sugar, but the *p*H of the neutral medium, on reaching its minimum value, displayed only a slight tendency to revert to neutrality. In estimating the KCl content of the yeast-extract media, it was found necessary to add an additional blank of 0·00044 g. for each 5 % of yeast water. The corrected KCl percentages are shown in Table 4.

Table 4

	Media		
Days of incubation	Neutral Czapek-Dox	Neutral Czapek-Dox plus 5 % yeast extract	Neutral Czapek-Dox plus 10 % yeast extract
	% residual KCl content of media (corrected)		
0	0·0497	0·0500	0·0496
3	0·0375	0·0434	0·0482
6	0·0354	0·0422	0·0452
9	0·0339	0·0405	0·0434
12	0·0315	0·0400	0·0425
15	0·0309	0·0350	0·0375
18	0·0309	0·0200	0·0202
21	0·0295	0·0154	0·0200
24	0·0250	0·0140	0·0173
Metabolized KCl	0·0247 %	0·0360 %	0·0323 %

The disappearance of KCl in the neutral medium is slower though more regular than in the acid medium. The yeast water decelerates the initial KCl metabolism up to the 12th day, after which the halide content drops rapidly until the 15th day. It seems reasonable therefore to assume that the activating effect of yeast extract is first directed towards the sugar metabolism to the detriment of the chloride, but that when the sugar has been eliminated, the activation is completely centred round the utilization of the KCl present.

The decreased rate of KCl metabolism during the early stages of growth definitely reduces the yield of pigment. In all three of the above inoculations, the amounts of recrystallized sclerotiorine obtained were only slightly in excess of 1 % of the dry mycelium weight.

SUMMARY

Growth of *P. sclerotiorum* on media containing KBr and KI in place of the usual KCl did not yield halide derivatives analogous to sclerotiorine, the ordinary

chlorinated metabolic product, while growth on media devoid of chloride in no way assisted the isolation of the red component. Variation of the KCl concentration showed that its elimination from the medium was quite regular after the initial period when the sugar metabolism was exceptionally rapid. Increased chloride concentration had no effect on the production of sclerotiorine and only affected the chlorine content of the other products of extraction of the mycelium. It is suggested that the pigment is a metabolic by-product rather than the fundamental end-product of the chloride metabolism. The activating effects of yeast extract was no more than equivalent to the effect of increased KCl concentration.

The authors desire to thank Prof. J. Reilly for his continued interest during the progress of this work.

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The Determination of 2:3-Diketo-*l*-gulonic Acid

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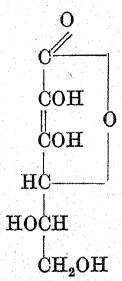
The first stage of the oxidation of *l*-ascorbic acid is of particular interest to the biochemist. The product, dehydro-*l*-ascorbic acid, retains its lactone ring and can be reduced to *l*-ascorbic acid by mild reagents such as H_2S, etc., in vitro, and can also be regenerated in vivo almost quantitatively. In aqueous solutions, dehydro-*l*-ascorbic acid undergoes mutarotation which is generally assumed to be due to the gradual opening of its lactone ring with the formation of 2:3-diketo-*l*-gulonic acid. This latter compound can still be converted into *l*-ascorbic acid by lactonization and reduction when treated with HI in vitro [Herbert, Hirst, Percival, Reynolds & Smith, 1933]. Regeneration apparently does not occur in vivo, since little if any antiscorbutic activity has been observed in solutions of dehydro-*l*-ascorbic acid which have undergone mutarotation for some time [Hirst & Zilva, 1933; Borsook, Davenport, Jeffreys & Warner, 1937].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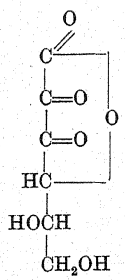
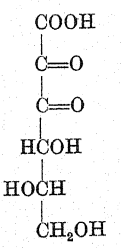

Dehydro-*l*-ascorbic acid


2:3-Diketo-*l*-gulonic acid

Whilst dehydroascorbic acid can easily be determined by titration of the regenerated ascorbic acid after reduction with H_2S, no direct method is available for the determination of diketogulonic acid. In this communication conditions are described under which this compound, which so far has not been isolated, can be determined directly.

Herbert *et al.* [1933] have shown that both ascorbic acid and freshly oxidized ascorbic acid give derivatives with 2:4-dinitrophenylhydrazine of m.p. 282° (decomp.) and 280° (decomp.) respectively. We have found that diketogulonic acid obtained by mutarotation from dehydroascorbic acid also gives with 2:4-dinitrophenylhydrazine a red derivative or derivatives which on solution in NaOH produce an intensely red colour. This colour reaction forms the foundation on which the procedure for the direct determination of the diketogulonic acid is based.

2:4-Dinitrophenylhydrazine reacts also with phenylpyruvic acid [Penrose & Quastel, 1937] and with other α-keto acids [Lu, 1939; Sealock & Silberstein, 1940; Sealock & Scherp, 1941], and consequently the reaction cannot be considered specific. In actual practice, however, it has been found that extracts of the tissues of normal guinea-pigs, and untreated human urine give a very low and fairly constant colour with dinitrophenylhydrazine; thus it should be possible to detect the appearance of even small quantities of diketogulonic acid by this reaction. Furthermore, the results suggest that the procedure outlined by us is open to further improve-

ments as regards specificity and sensitivity should the necessity arise.

GENERAL TECHNIQUE

Reagents. (1) 2 g. 2:4-dinitrophenylhydrazine (laboratory reagent) were dissolved with warming in 1 l. 2*N* HCl (AnalaR). The stock solution was filtered before use.

(2) *N* NaOH (AnalaR).

(3) *l*-Ascorbic acid, 99–100 % purity. Messrs Roche Products, Ltd.

(4) Dehydro-*l*-ascorbic acid was obtained by oxidizing *l*-ascorbic acid with the theoretical amount of 0·1*N* I_2 (AnalaR). Yield as determined by reduction with H_2S, 95–100 %. The solutions were used immediately after preparation.

(5) 2:3-Diketo-*l*-gulonic acid was prepared by allowing a solution of dehydro-*l*-ascorbic acid to mutarotate for 18 days at room temperature without neutralizing the HI formed in the preparation of the dehydroascorbic acid [cf. Herbert *et al.* 1933]. After this time the ascorbic acid regenerated from the solution by reduction with H_2S reached a value varying from 10 to 15 % of its original concentration, and this remained approximately constant for at least 10 days. The intensity of the colour given by the solutions with 2:4-dinitrophenylhydrazine increased during mutarotation and also became constant after about 18 days. Diketogulonic acid more than 10 days old was never used in this investigation.

Factors influencing the reaction. The intensity of the colour produced could be increased by raising the quantity of dinitrophenylhydrazine used in the reaction. The amount of the reagent that could be employed was, however, limited by the fact that when certain quantities of the hydrazine were exceeded the addition of the alkali made the reacting solutions turbid. For the range of concentrations of diketogulonic acid used by us the addition of 1 ml. of the hydrazine solution produced a colour of suitable intensity without influencing the transparency of the solution. The use of 2:4-dinitrophenylhydrazine (laboratory reagent) recrystallized from ethyl acetate did not influence the colour reaction in any way. The time and temperature of heating of a mixture of diketogulonic, dehydroascorbic, and ascorbic acids with dinitrophenylhydrazine greatly affected the intensity of the colour; this will be discussed in detail later.

As would be expected the quantity of NaOH which it was necessary to add depended on the quantity of acid present in the reacting solution, but under the above conditions 4 ml. of *N* NaOH amply satisfied the requirement, and an increase to 6 ml. was without influence on the colour production.

After the addition of the *N* NaOH a brown colour developed immediately, which rapidly turned to orange in dilute and red in more concentrated solutions. As the final colour is unstable it was necessary to measure its intensity at a given time soon after production.

Measurement of colour. The measurement was carried out in a Duboscq type of colorimeter. Owing to the instability of the colour it was, however, more convenient to have recourse to a photometric technique [King & Haslewood, 1937] instead of matching the colour directly. For this purpose a spectrum green colour filter (Ilford no. 604) was introduced into the eyepiece of the instrument and the standard was replaced by neutral density filters against which the absorption was measured. Although a range of these filters was employed all results were calculated for the 0·5 neutral density filter.

Effect of time and temperature on colour production. As already mentioned the time and temperature of heating diketogulonic, dehydroascorbic or ascorbic acids with dinitrophenylhydrazine greatly influenced the intensity of the colour production. This is probably due in part to the changes the last two compounds undergo to yield dinitrophenylhydrazine derivatives similar to, if not identical with, the derivative yielded by diketogulonic acid. The reaction was therefore studied after various periods of heating at 25°, 50° and 75°. To each of a series of tubes containing 9 ml. solution of the individual acids previously brought rapidly to about 2° above the desired temperature, 1 ml. of dinitrophenylhydrazine reagent was added and the tubes placed in the respective thermostats. Those mixtures which were submitted to high temperatures were lightly corked to avoid evaporation. The solutions were then shaken for a short time in order to adjust their temperature to that of the thermostat. At various intervals one of each of the series was withdrawn, cooled rapidly if necessary, treated with the alkali, diluted to 25 ml., and the colour measured as described above.

The results are given in Figs. 1, 2 and 3. On careful examination of these data it appeared to us that the colour produced after 1 hr. at 25° was the most suitable for the quantitative assessment of diketogulonic acid in the presence of the other two acids, for the following reasons. It will be seen from Fig. 1 that the intensity of the colour given by dehydroascorbic acid under these circumstances is only about 8 % of that given by its equivalent of diketogulonic acid and that this percentage increased with time. At 50° and 75° (Figs. 2 and 3) on the other hand the reaction of dehydroascorbic acid is increased in 1 hr. to as much as 44 % and 81 % respectively of that of its equivalent of diketogulonic acid. Furthermore at 25° ascorbic acid even in quantities as high as 2 mg. does not react at all. In addition it might be mentioned that as the reaction proceeded there was a tendency for the formation of a reddish precipitate, especially at the higher temperatures, which was

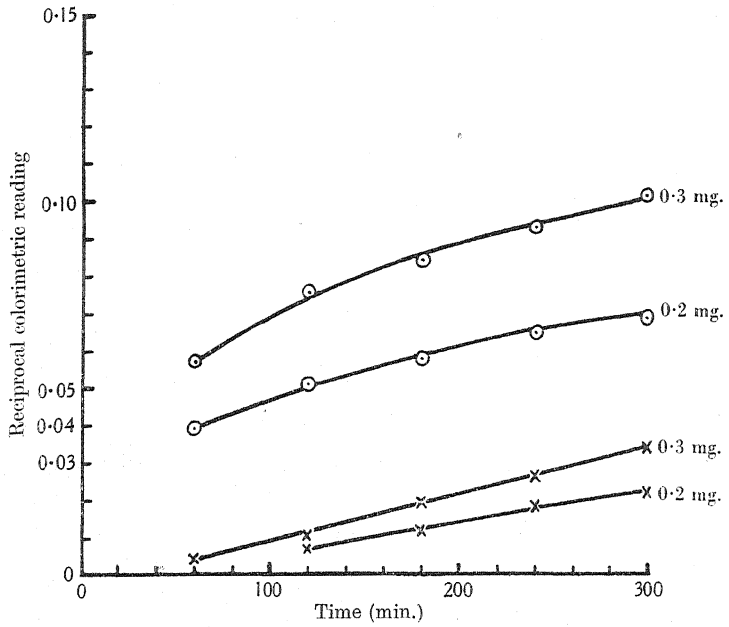


Fig. 1. Colour production at 25°. ⊙—⊙ diketogulonic acid. ×—× dehydroascorbic acid. In this and the following graphs, diketogulonic and dehydroascorbic acids are expressed as equivalent quantities in mg. of ascorbic acid from which they were prepared.

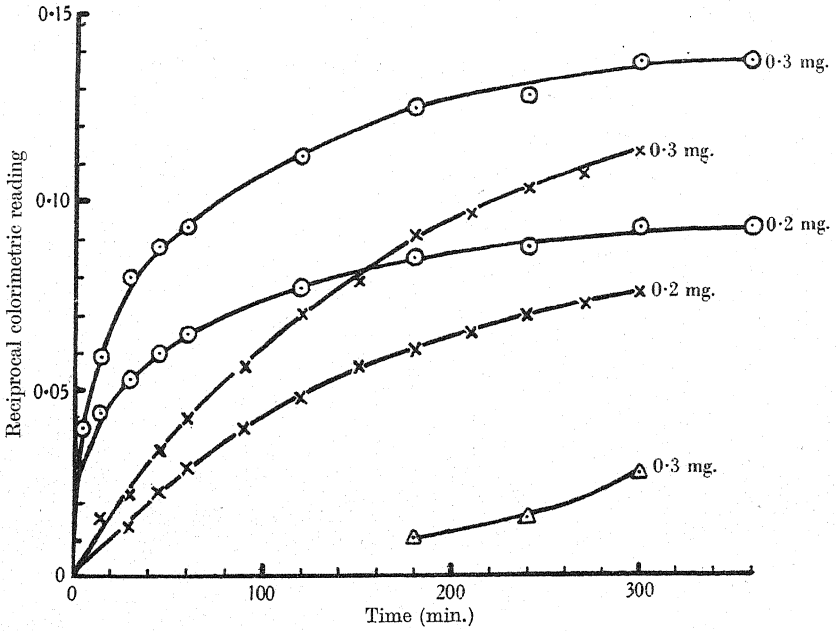


Fig. 2. Colour production at 50°. △—△ ascorbic acid. Other symbols are as in Fig. 1.

soluble in *N* NaOH. At 25° the amount of this precipitate was very small. It is also of significance that in all these experiments the colour produced at any time when expressed as a percentage of the maximum colour obtained at 75° after 300 min., was the same in the two concentrations both for dehydroascorbic acid and diketogulonic acid. This was also found to be true for ascorbic acid when it was possible to obtain significant figures namely, at 75°.

Calibration curve. Calibration curves for diketogulonic acid and dehydroascorbic acid covering a range of 0·05–0·5 mg. in the case of the former and 0·35–0·5 mg. in that of the latter, were constructed from the figures obtained by keeping the reaction mixtures for 1 hr. at 25°. For convenience the diketogulonic acid is expressed as mg. of ascorbic acid from which it was prepared, since an absolute value cannot be obtained owing to the fact that it has not been isolated. These units are plotted against the reciprocal of the reading on the colorimeter scale ($1/R$). The results are the mean of the figures obtained from three different solutions determined in duplicate; the variation of the individual readings from the mean were within the limits of ±3%. As can be seen from the curve (Fig. 4) the relationship between the concentration and the reciprocal colorimeter readings is linear up to about 0·4 mg. of diketogulonic acid; beyond that concentration there is a slight deviation from Beer's Law. Since the intensity of the colour produced by dehydroascorbic acid is only about 1/12 of that produced by diketogulonic acid it was not possible to obtain values lower than 0·35 mg. It is seen that figures for the colour given by diketogulonic acid in pure solution approximating concentrations as low as 1 part in 200,000 can be read off the calibration curve.

The curve does not pass through the origin. This can be explained in two ways. Either some absorp-

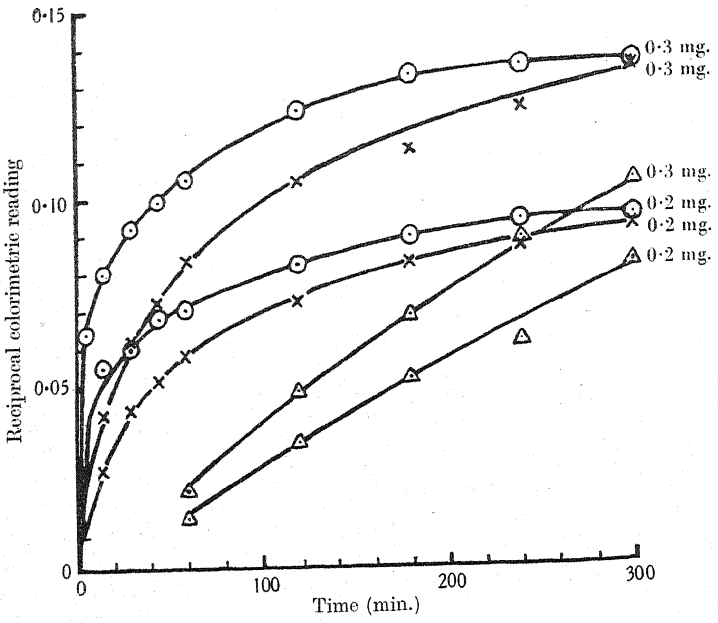


Fig. 3. Colour production at 75°. The symbols are as in Fig. 2.

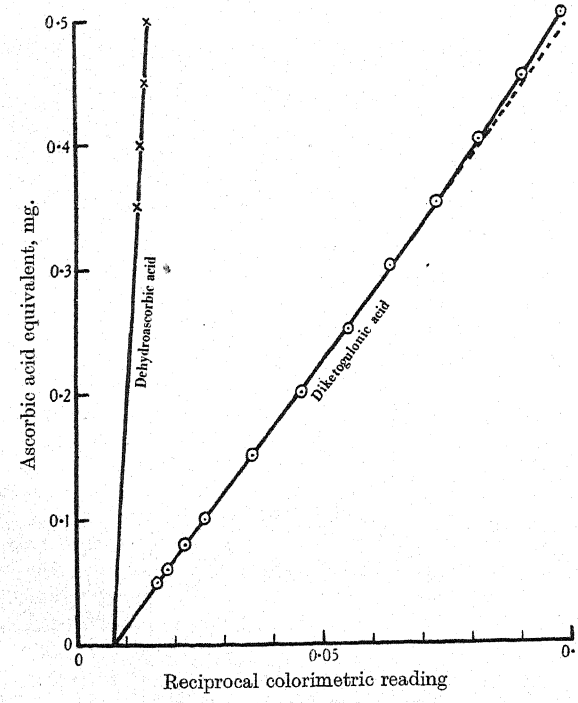


Fig. 4. Calibration curve.

tion is produced by the reagents or Beer's Law is not obeyed in very dilute solution. We favour the former explanation, since some experimental evidence was obtained supporting this view. By measuring the absorption of solutions of $KMnO_4$ with the spectrum green filter, a linear relationship between $1/R$ and the concentration of the $KMnO_4$ could be obtained, obeying Beer's Law even in dilute solution, i.e. the curve passing through the origin. When a blank solution containing 1 ml. of dinitrophenylhydrazine and 4 ml. *N* NaOH in 25 ml. was matched against a suitable dilution of $KMnO_4$ it was found that the value for the absorption of the concentration of $KMnO_4$ equivalent to the blank expressed in units of the 0·5 neutral density filter coincided with the zero value obtained from the calibration curve (0·007). All results including those given in Figs. 1, 2 and 3 were therefore corrected for zero displacement.

Procedure. A quantity of the solution containing 0·05–0·5 mg. diketogulonic acid is introduced into a 6 × 1 in. tube and the volume, if necessary, is made up to 9 ml. with distilled water. 1 ml. of the dinitrophenylhydrazine reagent is then added and the tube placed in a thermostat at 25° (± 0·2°) for 1 hr. At the end of this time 4 ml. of *N* NaOH are added, the solution is transferred to a 25 ml. graduated flask and after 1 min. is made up to volume with the washings and with distilled water. After mixing the contents the colour of the solution is measured in the colorimeter as rapidly as possible (30 sec.) in order to avoid any error due to fading. Six readings are taken which should not deviate by more than ± 2 % from the mean. The quantity of diketogulonic acid present is then read off the calibration curve.

If dehydroascorbic acid is present it is determined by reduction with H_2S and titration with indophenol and the colorimetric equivalent is obtained from the calibration curve. The calculation is then made as follows:

$$\frac{1}{R_y}=\frac{1}{R}-\frac{1}{R_x}+\frac{1}{R_c}.$$

R = Observed colorimetric reading.
R_x = Equivalent colorimetric reading for quantity of dehydroascorbic acid found.
R_c = Colorimetric reading due to blank (zero correction).
R_y = Colorimetric reading for diketogulonic acid.

No correction is necessary for ascorbic acid since no colour is obtained after an hour at 25° with quan-

tities less than 2 mg. and such high concentrations usually fall outside the range.

Table 1 gives the degree of accuracy that can be attained by determining diketogulonic acid in concentrations of about 0·5–5 mg. in 100 ml. solution.

Table 1. *Determination of diketogulonic acid in the presence and in the absence of dehydroascorbic and ascorbic acids*

Diketogulonic acid		Diketogulonic acid in the presence of dehydroascorbic and ascorbic acids			
		Dehydro-ascorbic acid	Ascorbic acid	Diketogulonic acid	
Added mg./100 ml.	Found mg./100 ml.	Added mg./100 ml.	Added mg./100 ml.	Added mg./100 ml.	Found mg./100 ml.
5·5	5·5	3·3	0	4·4	4·4
5·0	5·0	5·5	0	3·3	3·3
4·4	4·4	3·3	0	2·2	2·3
3·8	3·7	5·5	0	1·1	1·1
2·5	2·6	5·5	0	0·55	0·6
2·2	2·3	3·3	22·0	3·3	3·4
1·2	1·1	4·4	22·0	2·2	2·3
1·1	1·1	5·5	22·0	1·1	1·1
0·55	0·5	5·5	22·0	0·55	0·6

In this and in the following tables diketogulonic acid and dehydroascorbic acid are expressed as equivalent quantities in mg. of ascorbic acid from which they were prepared.

Effect of p*H on the colour production.* In studying the colour production in the presence of buffer solutions it was observed that the intensity of the colour produced by a given concentration of diketogulonic acid varied with the quantity of buffer added. This point is illustrated by the results given in Table 2. It was evident that the reason for the

Table 2. *Effect of buffering capacity on colour production*

Amount buffer, ml. in 9 ml. test solution containing 0·3 mg. diketogulonic acid	1	2	3	4	5
% Colour produced	102	108	113	114	122

Phosphate buffer *p*H 7·4; 2·72 g. KH_2PO_4 + 15·9 ml. *N* NaOH in 200 ml. solution

The colour intensity for 0·3 mg. diketogulonic acid obtained from the calibration curve = 100 %.

deviation in the colour production was the *p*H of the reacting solution after the addition of the dinitrophenylhydrazine. This difficulty can be overcome by adjusting the *p*H of the test solution to about 2. Table 3 gives the results of an experiment in which diketogulonic acid (0·3 mg./9 ml.) in a number of buffered solutions of various *p*H's as well as in solutions containing different quantities of HCl, was determined by the above procedure before and after the adjustment of the test solution to *p*H 2. It is seen that the intensity of the colour produced increased with the rise in the *p*H of the test solution.

Table 3. *Influence of* p*H of test solution on colour production*

Buffer or acid added	Quantity ml. in 9 ml. test solution	*p*H	Colour production % Before adjustment of *p*H	Colour production % After adjustment to *p*H 2
Phosphate-citrate buffer*	2	4·0	105	100
,, ,,	2	5·0	107	101
,, ,,	2	6·0	109	101
,, ,,	2	7·0	117	100
Borate buffer†	2	7·4	115	99
Phthalate buffer‡	2	6·0	107	101
N HCl	0·5	—	94	101
,,	1·0	—	88	100
,,	2·0	—	74	101

* McIlvaine [1921]. † Palitzsch [1915].
‡ Clark & Lubs [1916].

The colour intensity for 0·3 mg. diketogulonic acid obtained from the calibration curve = 100 %

As the results of the preceding experiment (Table 2) show, this difficulty does not hold if the buffering capacity of the test solution is low, since the addition of the phenylhydrazine reagent then brings about a *p*H similar to that which obtains in the pure diketogulonic acid solution, which is used as the standard for comparison, after the addition of this reagent.

DETERMINATION OF DIKETOGULONIC ACID IN BIOLOGICAL MATERIAL

Blood and plasma. In view of the observations made in the preceding section it became evident that in preparing trichloroacetic acid extracts of tissues for the determination of diketogulonic acid, the quantity of the acid present in the extract would have to be considered. Fortunately it was found that up to 4 ml. of 5 % trichloroacetic acid the intensity of the colour was not affected. An equal volume of 10 % trichloroacetic acid was therefore added to guinea-pig whole blood or plasma into which a known quantity of diketogulonic acid had been previously introduced. After removing the precipitate by centrifugation, aliquot portions of the

Table 4. *Determination of diketogulonic acid in whole blood and plasma*

Whole blood mg./100 ml.		Plasma mg./100 ml.	
Added	Found	Added	Found
2·5	2·37	2·5	2·4
5·0	5·0	5·0	5·4
10·0	9·5	10·0	10·2
15·0	14·3	15·0	16·0
20·0	20·4	25·0	24·0
33·0	32·5	34·0	35·0
40·0	39·2	50·0	49·5
66·0	64·0	67·0	68·0
100	101	80·0	82·0
200	196	100	100

extract, not exceeding 4 ml. were submitted to the procedure described above. As the extracts of the whole blood or of the plasma gave a small but significant colour (equivalent to about 2 mg./100 ml.) with dinitrophenylhydrazine, it was necessary to determine the intensity of this colour in a control experiment, so that a correction could be made. Table 4 gives 10 representative determinations. The standard error of percentage deviation in concentrations from 2·5 to 200 mg./100 ml. of blood or plasma was found by us to be $3\frac{1}{2}$ %.

Liver, kidney and muscle. The tissues obtained from normal guinea-pigs, the added diketogulonic acid and 2 parts by weight of 10 % trichloroacetic acid were ground with sand in a mortar, squeezed through muslin, reground, and centrifuged in the usual way. The sensitivity of the determination in this case was conditioned by the fact that the use of too much extract produced turbidity after the addition of the alkali. We nevertheless found that it was possible to determine 0·15 mg./g. of tissue with a standard error of percentage deviation of $4\frac{1}{4}$ % after correcting for the blank. Results chosen at random are given in Table 5. The approximate equivalents of diketogulonic acid yielded by the blanks were, muscle 0·1 mg./g.; kidney 0·15 mg./g.; liver 0·2 mg./g.

Table 5. *Determination of diketogulonic acid in liver, muscle and kidney*

Liver mg./g.		Muscle mg./g.		Kidney mg./g.	
Added	Found	Added	Found	Added	Found
0·15	0·14	0·15	0·15	0·15	0·16
0·20	0·21	0·20	0·21	0·20	0·21
0·25	0·26	0·25	0·26	0·25	0·23
0·50	0·51	0·50	0·52	1·00	1·04
0·75	0·75	0·65	0·64	1·00	0·98
1·00	1·02	1·00	0·95	1·50	1·56
1·00	0·98	1·00	1·04	2·00	1·98
1·25	1·30	1·25	1·30	2·00	2·09
1·50	1·60	1·50	1·52	3·00	3·00
1·50	1·60	2·00	1·94	3·00	3·15

Urine. The diketogulonic acid was added to freshly voided human urine and the determination was carried out directly on the urine without previous extraction. When necessary a correction was made for the blank. This was never higher than the equivalent of 5 mg. of diketogulonic acid per 100 ml. of urine. Table 6 gives some results obtained with concentrations 2·5–40 mg./100 ml. of urine. The standard error of percentage deviation of all the urine determinations carried out by us was 3 %.

Table 6. *Determination of diketogulonic acid in urine*

Added, mg./100 ml.	2·5	5·0	10·0	10·0	15·0
Found, mg./100 ml.	2·5	5·0	10·2	9·6	15·2
Added, mg./100 ml.	20·0	20·0	25·0	30·0	40·0
Found, mg./100 ml.	20·0	19·4	24·7	29·0	40·0

We should like to take this opportunity of expressing our gratitude to Dr R. G. Hatton, Director of East Malling Research Station, for laboratory accommodation. Thanks are also due to Messrs Roche Products Ltd. for a gift of ascorbic acid. One of us (J. R. P.) is indebted to the Medical Research Council for a whole time grant.

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Urea as a Partial Protein Substitute in the Feeding of Dairy Cattle

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The belief that certain simple nitrogenous compounds such as urea are capable of supplying to the ruminant a significant proportion of its nitrogen requirement is many years old. The theory most commonly advanced to account for the utilization of these simple compounds is that they are built up into protein by certain micro-organisms of the rumen which use them as a source of nitrogen for their own multiplication. The protein so formed is subsequently broken down in the small intestine to

amino-acids which are absorbed into the blood stream of the host along with those resulting from the digestion of the diet.

This theory has never been fully substantiated. Many feeding trials were carried out, mainly on the Continent, in the first three decades of the present century, but insuperable difficulties are encountered when an attempt is made to interpret the results of most of the experiments. In some instances the periods in which the non-protein nitrogen was fed were too short to enable reliable conclusions to be drawn. In others the periods were longer but the body-weights of the animals were not recorded, and again in others the controls were inadequate. These difficulties were emphasized by Krebs [1937] who, in reviewing the literature up to 1936, cited over 100 references and stressed the highly controversial nature of the results.

Since 1936 further publications have appeared suggesting that at least a portion of the dietary protein of ruminants can be replaced by substances such as urea. Hart, Bohstedt, Deobald & Wegner [1939] found that urea and ammonium bicarbonate, when added to a diet poor in protein and fed to growing calves, caused gains of live weight which were larger than those produced by the untreated diet, although the increases were slightly smaller than those obtained by the addition of the same amount of nitrogen in the form of casein. Bartlett & Cotton [1938] found no significant differences when one-third of the protein in the ration of young dairy heifers was replaced by urea, though they believed that the urea diet was slightly inferior to the protein diet. These publications, together with several others, have been discussed recently by Benesch [1941] and reviewed by Owen [1941]. It may be noted that they are concerned mainly with the effect of feeding on the growth of young stock or sheep, and that none of the work published in recent years has dealt with the value of these simple nitrogenous compounds for milk production.

The controversial nature of results and the war-time shortage of feeding-stuffs appeared to justify a thorough reinvestigation of the dietary value of urea for milk production. The general plan of attack and the objects in view have been outlined briefly by Owen, Smith & Wright [1941]. One section of the work was to consist of metabolism trials with lactating cows, in which urea would be compared with blood meal as a source of one-third of the nitrogen of the production ration. In the second section use was to be made of a steer with a large rumen fistula to determine by both in vivo and in vitro methods whether protein synthesis could actually be observed in the contents of the rumen and, if so, to study the factors affecting the magnitude and efficiency of such synthesis.

The present communication is limited to an account of the metabolism trials and their results.

OUTLINE OF THE EXPERIMENTS

Seven Ayrshire cows of average milk yield were used for the experiments. They were first fed a production ration in which a third of the nitrogen was supplied by blood meal, a product which has been shown by Morris & Wright [1933*a*] to contain a protein of first-class biological value for milk production. After the animals had been receiving this diet for a few weeks, the blood meal was replaced by its nitrogen equivalent of urea plus its energy equivalent as pure starch. With the first two cows the urea was fed for a period of 6 weeks and then replaced by blood meal. With the remaining five cows a period of about a fortnight without either urea or blood meal was inserted immediately after the first urea period. Each dietary change from one period to another was made gradually over 5 days. Further details as to the dates of the various periods and their duration are given in Table 1.

The animals were kept in special stalls in the metabolism house which has already been described by Morris & Wright [1933*b*]. The stalls were so fitted that by means of a continuously moving belt the urine and faeces of the cows could be collected separately with only slight mutual contamination. The faeces were weighed and sampled every 2 days, but the vessels for collecting urine were emptied each day and the urine sampled. Toluene was used to minimize bacterial action in these vessels. Composite 2-day samples of urine were then made for analysis. The slight contamination of the urine with faeces imparted to the urine a marked 'urease activity', with the result that part of the excreted urea was liable to be converted to ammonia in the collecting vessels. This rendered the estimation of urea itself relatively valueless and it was found necessary to estimate urea and ammonia nitrogen together. Preliminary tests showed, however, that this conversion of the urea did not result in any loss of nitrogen from the urine.

The milk yields were recorded daily, and with the first five cows composite 2-day samples were analysed for all the major constituents. With the remaining two cows the analysis was carried out at less frequent intervals. Urea and non-protein nitrogen were also determined at suitable times throughout the experiments in both the blood serum and the milk.

Two of the animals were weighed at the beginning and end of the experiment, and the remaining five at the beginning and end of each period.

The basis of rationing for maintenance was 5·5 lb. starch equivalent and 0·5 lb. protein equivalent per 1000 lb. live weight. For milk production the allowance was 2·5 lb. starch equivalent and 0·45 lb. protein equivalent per gal. The production ration per gal. of milk consisted of 0·25 lb. blood meal, 2 lb. oats, 1½ lb. bran, 0·25 lb. treacle and 0·5 lb. starch.

In Exp. 1 the maintenance ration for cows A and B consisted of 28 lb. swedes, 14 lb. straw and 2 lb. oats per day. It was found in this experiment that swedes were technically undesirable since they were not always completely consumed by the animals and since they contained a somewhat large proportion of non-protein nitrogen of which the nutritional value cannot at present be accurately assessed. In the experiment with cows C and G, a maintenance ration consisting of 13·5 lb. hay (6 % crude protein content) and 2·12 lb. potato starch per day was therefore fed, and the bran in the production ration was reduced by 0·5 lb. to compensate for the resulting slight increase of protein equivalent. In Exps. 1 and 3 a reduction was made in the production ration during the course of the work to parallel the decrease in milk yield. The point at which this change was made is shown in Fig. 1. In Exp. 2 the production ration was fed at the same level throughout.

The constituents of the production ration and the oats or starch from the maintenance ration were weighed each day and then thoroughly mixed in a mechanical mixer, the urea being dissolved in the molasses plus an equal volume of water. This procedure avoided any risk of localized concentrations of urea. The mixture was fed twice daily. There were no refusals of food during the urea periods, which indicates that the mixture was quite palatable. In Exp. 2 the hay was sampled and analysed at frequent intervals to ensure that the nitrogen intake was maintained at the correct level. The hay in Exp. 3 and the mixed diet in Exps. 2 and 3 were analysed every 2 days.

In all Kjeldahl determinations a mixed catalyst of potassium and copper sulphates and powdered selenium was used. Sulphur in the urine was determined by a modification of the method of Wolf & Osterberg [1910]. After having been boiled with conc. HNO_3 under a reflux condenser, the urine was subjected to a second oxidation with Denis's [1910] copper nitrate reagent. The total sulphur was then determined gravimetrically as $BaSO_4$. This method gave results agreeing well with those obtained by oxidation with a mixture of sodium carbonate and sodium peroxide. Urea and ammonia in the urine were determined by the urease method of Van Slyke and Cullen as described by Peters & Van Slyke [1932]. Before the determination of ammonia the samples of faeces were subjected to the action of urease.

RESULTS AND DISCUSSION

Milk yields

The milk yields for 2-day period for all seven cows are shown in Fig. 1, while the average yields in each period are recorded in Table 2. It will be seen from Fig. 1 that on feeding urea to cow A there was a decrease in yield from some 35 kg. to about 29 kg. during the first 16 days, a fall of 17 %. This decrease was, however, followed by a slight recovery, with

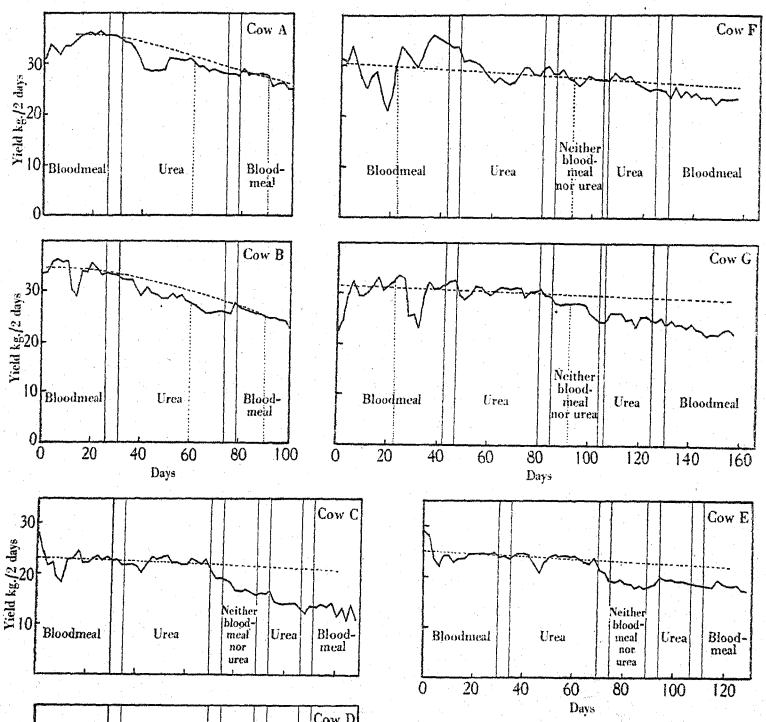

Fig. 1. The milk yields of the seven cows throughout the various periods. Each dietary change from one period to another was made in five stages over five days except for the third change with cows F and G which was made in two stages over two days. These change-over periods are represented by the pairs of vertical lines. In accordance with the decrease in milk yields, an appropriate reduction was made in the production ration fed to cows A, B, F and G at the points denoted by the vertical dotted line.

Table 1. *Plan of experiments*

Exp. no.	Period no.	Duration days	Description
1	1	26	Blood meal
Cows A and B	2	44	Urea in place of blood meal
6. xii. 39 to	3	28	Blood meal
19. iii. 40			
2	1	30	Blood meal
Cows C, D and E	2	36	Urea in place of blood meal
15. iv. 40 to	3	12	Neither blood meal nor urea
22. viii. 40	4	14	Urea replaced in the diet
	5	18	Blood meal
3	1	43	Blood meal
Cows F and G	2	29	Urea in place of blood meal
3. iii. 41 to	3	17	Neither blood meal nor urea
7. viii. 41	4	17	Urea replaced in the diet
	5	25	Blood meal

Table 2. *The average milk yields during the various periods (kg./2 days)*

	Cow						
Period	A	B	C	D	E	F	G
1	34·4	34·0	22·8	23·3	24·6	30·3	29·6
2	30·6	31·5	22·3	21·5	23·6	28·7	30·4
3	27·0	25·0	17·6	17·3	18·7	27·9	26·9
4	—	—	14·9	16·8	19·2	26·9	25·3
5	—	—	13·3	15·0	18·2	24·1	23·1

the result that during the last 3 weeks of urea feeding the yield approached closely to a value which might reasonably have been recorded as normal had blood meal been fed throughout. In fact the averages quoted for cow A in Table 2 suggest that urea did not result in any very serious decrease in yield, if the normal fall in lactation is taken into account. The urea feeding period in this experiment coincided with the exceptionally cold spell in the early part of 1940, and it appears that the temporary fall in yield may have been due to adverse weather conditions. With cow B no such initial decrease took place and the efficiency of urea for milk production appears to have approached very closely to that of blood meal.

The diagrams for cows C, D, E and G and the averages in Table 2 all show that milk production during the 4 or 5 weeks of urea feeding was practically as high as that obtained with blood meal, and that on removing urea from the diet a marked decrease in milk yield at once ensued. Thus with these four cows, yields of some 22, 21, 23 and 30 kg. at the end of period 2 were rapidly reduced to 17, 17, 19 and 27 kg. by the withdrawal of urea from the diet. These decreases amount to between 10 and 20 %. With cow F the milk yield in period 1 was so variable that it is difficult to estimate the effect of urea with any certainty, but this animal appears to have behaved like cow A in that there was an initial decline in yield followed by a partial recovery. On withdrawing urea from the diet, however, the yield in this instance did not decline appreciably. It will be shown later that of the two cows F and G which were observed at the same time under identical conditions, the wastage of the ingested urea was much greater with cow F than with cow G. This fact may explain why urea did not seem to be as effective with cow F as it was with G.

It will be observed from Fig. 1 and Table 2 that for cows C, D, E and G the replacement of urea, and subsequently of blood meal, in the diet, after the period in which neither was fed, did not cause the milk yield to return to its expected (normal lactation) value. This is not an unusual experience, for if the milk yield is once allowed to decrease through inadequate feeding it is most difficult, particularly in the latter half of the lactation, to increase it again merely by an improvement in the diet.

So far as milk yields are concerned it may therefore be tentatively concluded that of the seven cows, five were able to utilize urea for milk production with reasonable efficiency, though the results for the sixth cow (F) were not clear-cut. With the remaining cow (A) there was an initial fall in yield at the outset of urea feeding, but this only proved to be temporary, and was probably due to the severe weather conditions. It has been suggested [Kay, 1942] that the feeding of urea in more than very limited quantities may have a toxic effect. It is clear that in the present experiments no such effect occurred. Indeed, with four of the seven cows the yield was practically maintained at its initial level in spite of advancing lactation.

Body weights

The body weights, which are recorded in Table 3, were obtained by weighing each animal three times on one day and averaging the values so obtained.

Table 3. *Body weights in kg.*

	Cows				
	C	D	E	F	G
At beginning of 1st blood meal period	464	473	488	483	478
At end of 1st blood meal period	450	425	450	446	428
Change	−14	−48	−38	−37	−50
At end of 1st urea period	450	455	478	445	413
Change during this urea period	None	+30	+28	−1	−15
At end of period when neither urea nor blood meal was fed	448	436	454	435	402
Change during this period	−2	−19	−24	−10	−11
At end of 2nd urea period	449	464	457	434	410
Change during this period	+1	+28	+3	−1	+8
At end of 2nd blood meal period	479	489	479	419	402
Change during this period	+30	+25	+22	−15	−8

Cows A and B were weighed only at the beginning and the end of Exp. 1. Their initial weights were 442 and 410 kg. respectively and their final weights were 394 and 409 kg.

The three weighings were made at 6 a.m., noon and 6 p.m. This precaution was taken to ensure that the average weight recorded for any one day was affected as little as possible by the ingestion of food and water or by the excretion of faeces and urine. Except on one occasion the differences between any of the three weighings on one day never exceeded 17 kg. and were usually much less.

It will be seen that the weights of all five cows in Exps. 2 and 3 decreased slightly during the first blood meal period, but that during period 2, when urea was substituted for blood meal, this tendency was checked, and in two instances (D and E) the decrease was converted to an increase. When, however, urea and blood meal were both withheld (period 3), the weights of cows D and E again showed a tendency to decrease in spite of the fact that the period was short and that it was accompanied by a reduction in milk yield. When urea feeding was resumed in period 4 these decreases in weight were again checked, and in period 5, except in the case of cows F and G, they were converted to gains.

It may be concluded that the urea feeding did not cause any significant loss of body weight, and that in certain instances gains were observed during the urea periods.

Nitrogen excretion

At 2-day intervals throughout the experiments composite samples of urine and faeces were analysed for total nitrogen and urea + ammonia nitrogen, and for creatinine and creatine. In the case of cows A and B urinary sulphur was also determined. Owing to the need for economy in space it is impossible to present here the large number of results obtained in this section of the work. Details of two typical examples are given in Fig. 2. Cow A gave similar results to cow B, while the results for cow C were representative of the remaining five animals. The average values for the intake and output of nitrogen and for the urea + ammonia excretion for Exps. 2 and 3 are shown in Table 4. It will be relevant to discuss at this point the metabolic data.

Nitrogen balances

It will be seen from Table 4 that, except in period 3, when both urea and blood meal were withheld, the animals appeared to be in a state of positive nitrogen balance to a somewhat remarkable degree. This was particularly true for cows C, D and E, the apparent positive balances attaining in some instances a figure of 90 g./2-day period. In view of the relatively small changes in the body weights of the animals (Table 3) it is difficult to regard these values as true indications of the precise state of nitrogen balance. We are unable to explain the tendency for our technique apparently to indicate such large positive balances. Each step of the procedure has been examined in detail with a view to solving this problem, but so far no solution has been found. It may be noted that in the work of Morris & Wright [1933*b*] and of Morris, Wright & Fowler [1936], in which the same technique was used, positive balances of as much as 50 and over 70 g. nitrogen per 2-day period were en-

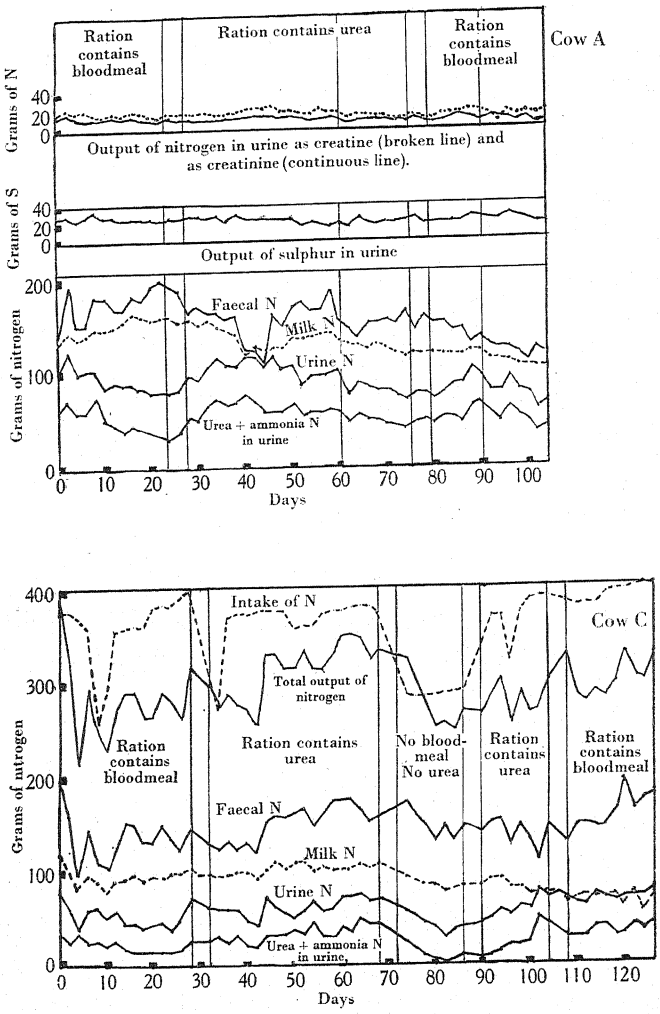

Fig. 2. Output of total nitrogen in milk, faeces and urine, output of nitrogen as urea + ammonia in urine, and total intake of nitrogen.

countered in extreme instances, whereas negative balances occurred infrequently and were usually very small.

We are, however, of the opinion that the figures recorded for the balance in any one period can be compared in a relative sense with the corresponding figures for other periods. Thus with cow C the figures of +76·9, +53·2 and +11·4 for periods 1, 2 and 3 indicate that a probable positive nitrogen balance when blood meal was fed was slightly reduced when the blood meal was replaced by urea and was very much further reduced (if not rendered negative) when neither urea nor blood meal was ingested. It seems reasonable to apply a similar method of interpretation to all the nitrogen balance figures in Table 4.

It was again restored, however, when urea feeding was resumed.

The fact that the nitrogen balance was so much more positive in the two main urea periods than in the intervening nitrogen-deficient period indicated either that the urea-nitrogen was retained and anabolized by the animals or that the presence of urea in the diet in some way rendered the other nitrogenous constituents of the food more readily available for the animals' use. In this latter connexion a study of the values recorded in Table 4 for the faecal nitrogen of periods 1 and 2 does not suggest that the amount of nitrogen absorbed by the animal for metabolic purposes from the other constituents of the food was greater during urea feeding than it was with blood meal. In other words the apparent

Table 4. *Nitrogen excretion (g. nitrogen/2 day period)*

Period	Urine	Faeces	Milk	Total	Nitrogen intake	Nitrogen balance	Urea + NH_3-N in			Creatinine g./2 days	Creatine g./2 days
							Urine	Faeces	Total		
Cow C											
1	52·0	136·1	94·9	283·0	359·9	+76·9	21·4	10·3	31·7	11·1	10·0
2	61·5	150·6	101·8	313·9	367·1	+53·2	32·9	15·6	48·5	10·3	5·8
3	42·7	147·6	84·1	274·4	285·8	+11·4	10·7	7·0	17·7	12·2	2·2
4	61·2	139·4	80·8	281·4	370·2	+88·8	25·3	10·5	35·8	13·3	5·8
5	68·8	161·8	67·4	298·0	390·4	+92·4	35·0	11·1	46·1	13·0	2·2
Cow D											
1	36·8	167·5	112·1	316·4	405·0	+88·6	18·3	17·9	36·2	6·2	7·8
2	71·1	172·5	109·1	352·7	404·0	+51·3	49·3	22·4	71·7	7·5	10·8
3	43·9	152·3	93·1	289·3	287·7	− 1·6	10·6	12·2	22·8	12·3	9·2
4	72·9	138·8	95·0	306·7	398·8	+92·1	45·9	12·2	58·7	9·0	12·5
5	76·1	164·9	88·0	329·1	418·8	+89·7	48·9	14·3	63·2	8·6	7·8
Cow E											
1	57·8	166·5	103·0	327·3	422·1	+94·8	30·4	19·5	49·9	11·7	8·7
2	90·3	166·8	108·3	365·4	406·6	+41·2	57·6	18·1	75·7	12·6	10·1
3	51·9	152·2	85·5	289·6	301·8	+12·2	11·4	9·0	20·4	16·1	7·3
4	102·2	132·2	92·8	327·2	424·0	+96·8	63·2	11·0	74·2	16·3	9·9
5	98·0	162·2	91·4	351·6	450·0	+98·4	60·8	12·8	73·6	12·7	5·4
Cow F											
1	96·0	162·0	120·9	378·9	388·1	+ 9·2	50·1	12·5	62·6	15·3	11·1
2	143·6	146·8	120·9	411·3	433·9	+22·6	95·8	15·5	111·3	14·9	15·9
3	59·4	127·2	115·0	301·6	298·2	− 3·4	24·8	6·9	31·7	14·5	10·3
4	88·1	133·6	117·8	339·5	356·5	+17·0	52·9	11·2	64·1	15·1	16·3
5	72·7	165·4	105·9	344·0	368·9	+24·9	34·8	9·4	44·2	14·3	11·3
Cow G											
1	67·4	174·0	131·9	373·3	398·3	+25·0	40·3	19·4	59·7	9·3	10·2
2	93·1	162·9	139·4	395·4	436·0	+40·6	57·1	21·9	79·0	9·1	12·8
3	44·2	139·6	123·7	307·5	302·9	− 4·6	15·7	9·8	25·5	8·5	10·6
4	71·3	138·3	122·8	332·4	365·0	+32·6	39·2	16·6	55·8	11·8	16·4
5	45·3	175·8	112·8	333·9	364·7	+30·8	16·9	14·3	31·2	9·6	8·6

If this method of interpretation is valid, two main conclusions may be drawn. (1) With three cows (C, D and E) the positive nature of the balance in the first urea period was significantly less than in the first blood meal period. In the case of the other two cows (F and G) the reverse was true. On the other hand, with all five animals the positive nature of the balance was very considerably reduced during period 3, when neither urea nor blood meal was fed.

utilization of urea cannot be ascribed solely to an increase in the digestibility of the other constituents of the urea ration.

Krebs [1937] has stated that an apparent retention of urea such as has been found here might merely be due to a swamping of the tissues with urea and not to a conversion of urea to protein. But if this were true one would expect urea to be equally effective in the diet of both the ruminant and the non-

ruminant animal, whereas it has been well established that in the latter ingested urea is of no nutritional value. Moreover, as a simple calculation will show, it is unreasonable to suppose that the large retention of urea-nitrogen observed in the present experiments could ever be explained by a swamping of the tissues with urea. If it be assumed that the amount of water in which urea can dissolve in the body of the cow is 75 % of its body weight and that the concentration of urea in this water is the same as that in the blood serum, for which the maximum value found in this work never exceeded 15 mg. urea-N/100 ml. serum, then a 500 kg. cow would retain only about 120 g. urea. But in the whole of the second period of these experiments the total amount of urea fed to each cow was of the order of 3000 g. Even if the level of urea in the tissues could rise well above that in the blood, it is difficult to believe that it could ever attain a figure which would be sufficient to explain the observed findings, nor would such an hypothesis be capable of accounting for the maintenance of the milk yield and body weights.

Creatinine and creatine

Judging from recent publications by Bloch & Schoenheimer [1941], who find that urea is not a precursor of creatine, and by Beard, Espenan & Pizzolato [1939], who claim that it is a precursor, any variations which might be found in the amount of creatinine and creatine excreted in the urine of the ruminant, either on a blood meal or urea diet, would be difficult to interpret. It was, however, felt that a study of the creatinine-creatine excretion might give some useful information regarding the metabolism of the animals ingesting urea. The urinary excretion of creatinine and creatine was therefore estimated for all seven animals by the usual colorimetric procedure of Folin. For the first two cows the coloured solutions were compared with the standard in a visual colorimeter. For the others a 'Spekker' photo-electric absorptiometer was used. The results for cow A, which were very similar to those for cow B, are shown in Fig. 2. The average values for cows C to G are recorded in Table 4. It is impossible to draw any definite conclusion from the results, for the variations from one 2-day period to another were usually too large to enable inter-period variations to be detected. Nor was there any appreciable change when both urea and blood meal were withheld. It seems probable that really substantial inter-period changes in these values can only be observed with cows subjected to relatively severe inanition such as that described by Morris & Ray [1939]. Further work on this subject is, however, clearly desirable, since the results do not fall into line with those published earlier by Morris & Wright [1933 *a*, *b*].

Diuresis

Since urea is a diuretic agent it is important to determine whether urea-feeding with ruminants is liable to cause excessive and harmful diuresis. The average weight of urine excreted per 2-days in the various periods is shown for all seven cows in Table 5.

Table 5. *Average weight of urine excreted in the various periods (kg./2 days)*

	Cows						
Period	A	B	C	D	E	F	G
1	15·36	9·63	6·28	5·25	5·25	12·89	8·62
2	12·99	10·55	7·11	11·96	9·62	12·65	9·68
3	12·75	8·32	7·31	9·01	7·54	8·73	7·55
4	—	—	9·05	12·51	14·35	7·83	8·22
5	—	—	8·88	10·28	9·34	7·80	6·72

With cows A and F there was no diuresis when urea replaced blood meal in the diet. With the other cows, however, in which the weight of urine was initially much less, increased excretion did occur during the urea periods. This was most marked with cow E, in which a value of 5·25 kg. in period 1 rose to 9·62 kg. in period 2. Again, the value of 7·54 kg. in period 3, when both blood meal and urea were withheld, increased to 14·35 kg. in period 4, when urea feeding was resumed. On the other hand, it should be noted that the figure of 9·62 kg. for the first urea period did not differ materially from that of 9·34 kg. for the final blood meal period, nor does the highest value of 14·35 kg. represent an abnormal excretion for a cow in 2 days. Smaller amounts were excreted in period 1 by cows A and F and again in period 5 by cow A. It therefore seems unlikely that urea, if used in a prepared production ration in which it accounted for some 30 % of the nitrogen, would induce any harmful diuresis.

The non-utilization of a portion of the ingested urea

The results recorded in Table 4 show that the total amount of nitrogen excreted in the urine each 2 days during the urea periods was greater than that excreted during the blood meal periods, and that this excess could be accounted for mainly by an increase in the excretion of urea + ammonia-nitrogen. With cow D, for example, a value of 36·8 g. for the total urinary nitrogen during the blood meal period increased to 71·1 g. during the urea period, an increase of 34·3 g., the corresponding values for urea + ammonia-nitrogen in the urine being 18·3 and 49·3, a difference of 31·0 g. If, however, it were assumed that urea was quantitatively converted to protein in the rumen and that this protein became available to the animal to the same degree as blood meal, the excretion of urinary nitrogen would be expected to remain the same in the urea periods as it was in the blood meal periods. It seems probable therefore that

Table 6. *The proportion of ingested urea which does not appear to be utilized by the animal compared with the level of urea in the blood*

Cow	Urea-N ingested in period g./2 days		The amount by which the urinary N in period 2 exceeded that in period 1		The amount by which the urinary N in period 4 exceeded that in period 5		% of ingested urea which appeared to be wasted in period		Average value for urea-N in the blood serum mg. N/100 ml. during period		
	2	4	Total urinary N g./2 days	Urea + NH_3-N g./2 days	Total urinary N g./2 days	Urea + NH_3-N g./2 days	2	4	1	2	% increase
A	85·7	—*	13·0	13·7	—	—	15–16	—	4·5	8·3	84
B	91·0	—	23·0	14·1	—	—	15–25	—	6·3	10·5	67
C	85·9	93·7	9·5	11·5	− 7·6	− 9·7	11–13	None	4·7	5·8	23
D	93·7	93·8	34·3	31·0	− 3·2	− 3·0	33–36	None	4·5	7·3	62
E	99·6	99·7	32·5	27·2	+ 4·2	+ 2·4	27–33	2–4	6·2	9·0	45
F	99·4	81·6	47·6	45·7	+15·4	+18·1	46–48	19–22	—†	—	—
G	99·8	83·6	25·7	16·8	+26·0	+22·3	17–26	27–31	—	—	—

* There were only three periods with cows A and B.
† No blood analysis was carried out for cows F and G.

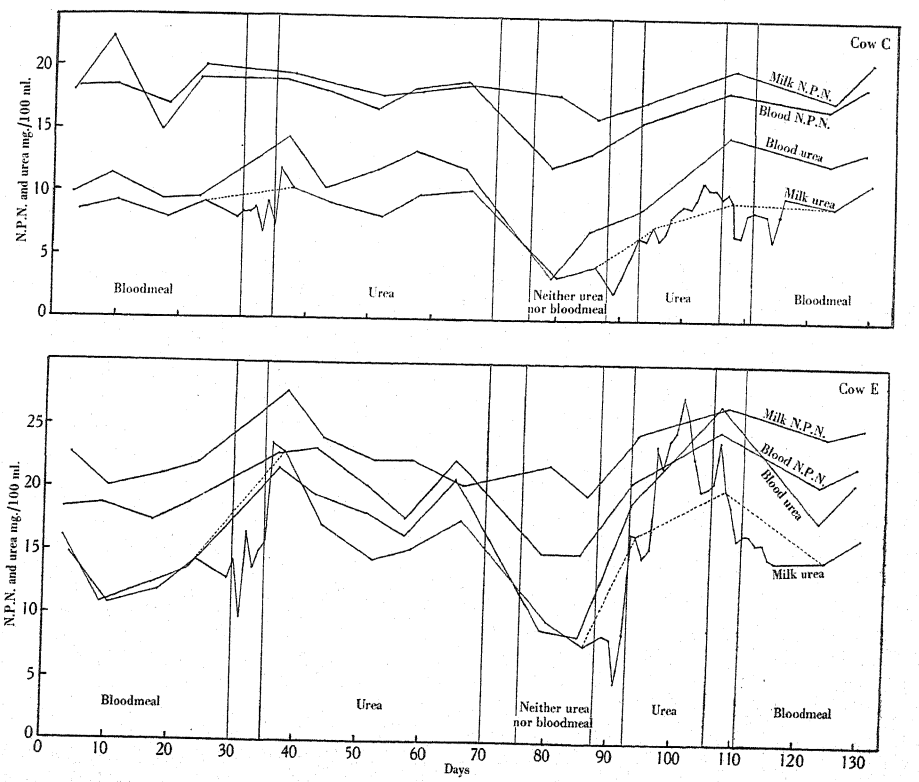

Fig. 3. The concentration of N.P.N. and urea in the blood and milk. For the five animals for which these data are available, cow C showed the least and cow E the greatest variation on replacing blood meal with urea. For this reason the results for the two cows have been chosen for illustration. The broken lines bring out the general parallelism between the concentration in the blood and that in the milk.

this increase in the nitrogen excretion when urea is fed may be taken as a measure of the amount of ingested urea which passes through the animal without acting as a protein substitute. In Table 6 the approximate amount of this unused urea is calculated. It should be noted that the value obtained for any one cow is roughly the same whether the total urinary nitrogen or only that excreted as urea + ammonia is used as the basis of calculation.

If the above reasoning is sound it may be concluded that the proportion of urea utilized varies greatly with different animals. With the seven cows

in the present work the proportion of urea passing through the animal unused in period 2 varied from 12 to 47 % and averaged 25 %. Moreover, it is important to observe that cow C, which showed the smallest wastage of urea, also showed the greatest benefit from the feeding of this substance, for the milk yield of this animal was maintained at as high a level in period 2 as in period 1, though removal of both blood meal and urea resulted in an abrupt decline. Again, cow F, which showed the least benefit in milk yield from urea feeding, also showed the greatest degree of wastage.

One further point should be noted. For cows C, D and E the values calculated for the wastage of urea in period 2, which followed a blood meal period, was very much higher than those calculated for period 4, in which urea feeding followed immediately after a period of 2 weeks during which both blood meal and urea were withheld. In fact, with cows C and D the nitrogen of urea appeared to be better utilized during period 4 than was that of blood meal fed in equivalent amounts during period 5. It appears from this fact that the proportion of the dietary urea which is wasted may depend to some extent on the nutritional state of the animal prior to urea feeding, a phenomenon which is not uncommonly encountered in other types of nutritional study. The same feature was observed with cow F, though not with cow G. It should be pointed out that these wastage values have been calculated on the assumption that the nitrogen of blood meal itself is completely utilized; they are therefore relative and not absolute.

Urea in the blood and milk

At intervals during the experiments urea and non-protein nitrogen were determined in the blood serum and milk of cows A to E and also in the milk of cows F and G. Typical results (those for cows C and E) are shown in detail in Fig. 3. It will be seen that the changes brought about by alterations in the diet were much smaller with cow C than with cow E. The average values of the blood urea for cows A to E during the first two periods are recorded in Table 6. During the urea period these exceeded those found during the blood meal period by amounts varying from 23 to 84 %. When both urea and blood meal were withheld from the diet a marked decrease in blood urea occurred (Fig. 3), and this was again followed by a substantial increase when urea feeding was resumed.

In all instances in which analyses of this type were obtained, there was a general parallelism between the concentration of N.P.N. in the blood and that in the milk, while the concentration of urea showed a similar relationship. It may be noted that the blood samples were taken within a period of minutes whereas the milk samples were representative of a complete 24 hr. secretion. The results confirm the earlier finding of Peskett [1934] that the urea content of cows' milk is almost identical with that of the blood.

In addition to these analyses, which were carried out at 5–10-day intervals, urea was also estimated on composite daily samples of the milk just before, during and after the change-over from period 1 to period 2, and also throughout period 4. Typical results are shown in Fig. 3. When urea was included in the diet immediately after blood meal feeding, the urea in the milk reached a maximum after 2–3 days. On the other hand, after period 3, when the diet was deficient in nitrogen, the time required for the same maximum to be attained was 8–10 days after the resumption of urea feeding.

Of the five cows for which blood urea values are available, the one which showed the least increase in the level of urea in period 2 (cow C) also showed the

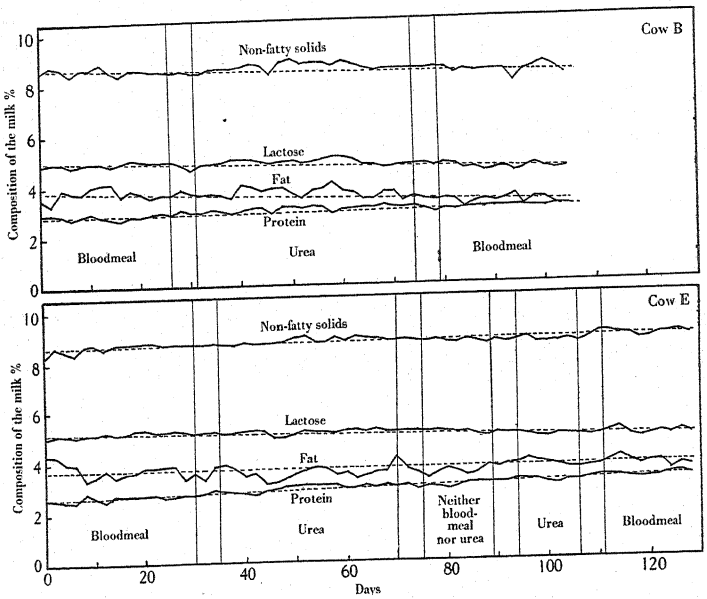

Fig. 4. The level of the major constituents of the milk throughout the various periods. The results for cow A were very similar to those obtained for cow B. The results for cow E are typical of those obtained for all the other five animals. In each instance the average values for the first and last periods have been plotted at points mid-way through these periods. The broken lines join these points.

least apparent wastage, and therefore the greatest utilization of this substance (Table 6). As regards milk yield (Fig. 1 and Table 2), this cow also benefited most from the inclusion of urea in its diet and suffered most from its withdrawal. For the other animals no definite correlation could be detected between blood urea levels and the apparent wastage of urea.

The composition of the milk

To both the producer and the consumer of milk it is important that data should be available to show whether the composition of the milk is adversely affected by the ingestion of urea. In the present experiments the highest concentration of urea in the milk was roughly 28 mg./100 ml., or 0·16 g. per pint (cow D, period 4); with the remaining cows it was very considerably below this level throughout most of period 2. Such an amount of urea is without effect on the taste of the milk and could not have any adverse effect on the consumer.

The principal constituents in composite 2-day samples of the milk were also determined for the first five cows, while for the remaining two similar determinations were made at less frequent intervals throughout the experiment. Two examples, which are typical of all the results, are shown in Fig. 4. They demonstrate clearly that urea-feeding did not bring about any alteration in the general composition of the milk.

SUMMARY

1. The value of urea as a partial substitute for dairy cows has been investigated by feeding it to seven lactating animals in an amount equivalent to 33 % of the nitrogen in the production ration or to 25 % of the total nitrogen intake. Data for milk yield, body weight, nitrogen balance and milk composition obtained while feeding this diet have been compared with the corresponding data obtained when the animals were receiving (*a*) blood meal and no urea and (*b*) neither blood meal nor urea.

2. The milk yields of five of the seven animals were well maintained when blood meal was replaced by urea. With four of the five cows under test a rapid and significant decrease in milk yield took place when urea was removed from the food. For one of the remaining two cows the graph of the milk yield was difficult to interpret owing to an initial decrease during the first few days of urea feeding. This decrease may have been due in part to the exceptional weather conditions then prevailing. The seventh cow did not appear to benefit to any significant extent from the inclusion of urea in its food.

3. Changes in body weights were not marked at any stage of the experiment, but on the whole they seemed to be better maintained on urea than on blood meal.

4. The nitrogen balance and excretion data show that, although urea was partially retained by all the animals, its retention was not complete. Compared with blood meal, amounts varying from some 12 to 47 % and averaging 25 % of the ingested urea apparently passed through the animal without being utilized. This apparent wastage was much reduced when urea feeding was preceded by a period in which the diet had been deficient in total nitrogen. The quantitative importance of this failure to utilize all the dietary urea is discussed.

5. The urea content of the blood tended to increase at the outset of the urea period and then to return towards its initial value as the urea feeding progressed. The urea content of the milk closely approximated that of the blood and never exceeded 28 mg./100 ml. (0·16 g. per pint), an amount which would not have any deleterious effect on the consumer.

One cow showed very little change in the urea content of its blood and milk at any time during the experiment. This animal also appeared to utilize the ingested urea to the greatest degree and to benefit most from its inclusion in the diet.

6. The ingestion of urea had no measurable effect on the percentage of protein, fat, lactose or total solids in the milk.

The authors wish to record their thanks to Dr M. P. Applebey and Prof. A. C. Chibnall, F.R.S., for their interest and encouragement during the course of the work, and to Imperial Chemical Industries Limited for a grant towards the expenses.

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The Application of the Spekker Photoelectric Absorptiometer to the Determination of Vitamin C

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In a footnote to their recent paper on the reliability of the chemical estimation of vitamin C by titration with 2:6-dichlorophenolindophenol, Harris & Olliver [1942] mention the application of a procedure involving the addition of an excess of dye and instantaneous measurement of the excess of colour, in order to differentiate the rapid reduction effected by ascorbic acid from the slower reduction due to non-specific reductants. Several methods employing this principle have already been described [Meunier, 1937; Guthe & Nygaard, 1938; Evelyn, Malloy & Rosen, 1938; Bessey, 1938; Bukatsch, 1939]; in every case, however, the authors have relied on measurements made on a single mixture of test solution and dye. The measurement is complicated by the fact that, in the presence of non-specific reductants, the colour is fugitive owing to the progressive decolorization of the excess dye by the interfering materials. Meunier [1937] investigated kinetically the decolorization of 2:6-dichlorophenol-indophenol by ascorbic acid, glutathione and cysteine and concluded that in the presence of the latter substances the loss of colour due to ascorbic acid could be satisfactorily assessed by making a plot of residual colour against time and extrapolating back to zero time. Subsequently other workers have also employed this device to allow for the fugitive character of the excess dye coloration.

When a Spekker photoelectric absorptiometer was employed for the determination of ascorbic acid in fruits and jams by this method it was found that fading of the colour of excess indophenol was sometimes very rapid, especially with solutions which had been treated with hydrogen sulphide for the estimation of the partially oxidized dehydroascorbic acid [McHenry & Graham, 1935]. Accuracy of extrapolation in such cases was not high and for this reason it was considered inadvisable to rely on readings of the colour due to a single addition of indophenol. An improved method, involving readings on a number of solutions with different amounts of excess indophenol, has therefore been developed.

METHOD

To a series of increasing amounts of a solution of 2:6-dichlorophenolindophenol, each diluted with distilled water to a fixed volume, are added uniform aliquot portions of the test solution. The colour of each mixture is measured by means of a Spekker photoelectric absorptiometer, a further similar aliquot of the test solution diluted to the same volume with distilled water only being used as a standard. The reading on each mixture is corrected for fading by making three observations over about a minute and extrapolating back to zero time. The colour values of the mixtures after correction are then plotted against the amount of indophenol reagent added; the appearance of excess reagent is marked by a sudden increase in the slope of the resulting curve, the point of flexure corresponding with the end-point of the titration.

Solutions employed. (*a*) *Indophenol reagent.* A solution of 2:6-dichlorophenolindophenol containing 0·25 mg./ml. was found to be most suitable for the Spekker titration. To stabilize the dye 10% of Clark's phosphate buffer of *p*H 7·0 was incorporated. This reagent was stored in a dark bottle in the refrigerator; it was renewed after about a month and standardized every 2 or 3 days in the usual manner [Harris & Olliver, 1942].

(*b*) *Sample solution.* The sample solution, which may be prepared in any suitable manner, should contain preferably from 5 to 8 mg. and in any case not more than 10 mg. ascorbic acid per 100 ml.

Titration procedure. The Spekker instrument measures the residual light transmission of the test solution and in examining a series of solutions of progressively increasing colour the instrument reading diminishes continually. The instrument must therefore be adjusted to allow of a suitable range of readings being obtained before complete extinction is reached. To this end 4 ml. of test solution are added from a pipette with a rapid delivery to 4 ml. of distilled water in a 1 cm. Spekker cell and mixed in with a quick shake. The instrument is adjusted to give a reading of 1·000 on this mixture with the Spekker no. 6 light blue filter in position. If the sample solution is itself of a red colour, it may be found that the sensitivity of the instrument with the light blue filter is very low. The sensitivity can be improved by substituting a green or even a red filter, but the titration curve obtained is of much less satisfactory shape. It is preferable to retain the blue filter and balance the instrument as accurately as the reduced sensitivity will permit.

The mixture in the cell thus prepared is retained as a 'zero' check.

Readings are then made on a series of solutions to which progressively increasing amounts of indophenol reagent have been added, the amount of water being correspondingly reduced to keep the total volume at 8 ml. in each case. The end-point of the titration must be known approximately, either from a preliminary titration or from previous experience with the particular material under examination, and the additions of indophenol reagent should be chosen to allow at least two additions before the end-point is reached. After the end-point, at least five further additions should be made, the amount of indophenol reagent being increased by 0·2 ml. each time. The observations on these solutions are made in the following manner.

To the appropriate indophenol reagent-water mixture in a second 1 cm. Spekker cell are added 4 ml. of test solution from the pipette previously used and at the same time a stop-watch is started. The solutions are mixed with a quick shake and the instrument reading is observed as rapidly as possble; the reading and time are noted. The instrument is thrown out of adjustment and readjusted, and a second reading and time, and finally a third, are obtained in the same way. These readings should be made as rapidly as possible, especially if appreciable fading, indicated by a steady increase in the instrument reading, is observed; all three readings should be completed within a minute at the most. In difficult cases a convenient device to enable the first reading to be obtained rapidly is to set the instrument to the approximate reading expected, as judged from the previous readings, before adding the test solution to the indophenol reagent-water mixture. The 'zero' reading should be checked after the observations on each mixture.

Interpretation of results. A plot is first made of Spekker reading against time for each mixture examined and the curves so obtained are extrapolated back to zero time to indicate the probable initial Spekker reading of each mixture. A second plot is then made of the extrapolated initial Spekker readings against the amount of indophenol solution added for the whole series of mixtures examined. This second plot represents the variation in colour of the sample solution, diluted to a fixed volume, as progressively increasing amounts of 2:6-dichlorophenolindophenol are incorporated. It comprises two relatively straight portions: one, horizontal, corresponds to the more or less uniform colour of the mixtures in which all the colour of the added indophenol has been discharged by the ascorbic acid present; the other, inclined fairly steeply, corresponds to the progressively increasing colour of the excess indophenol. These straight portions intersect at a relatively sharp angle and the point of intersection corresponds with the end-point of the titration.

The accuracy of the determination of the point of intersection depends primarily upon the correct drawing of the steeper portion of the plot. In practice the individual results deviate somewhat from linearity owing to random fluctuations; it was thought possible, therefore, that visual assessment of the optimum straight line to represent these results might not be sufficiently exact to avoid the introduction of appreciable errors. This point was examined by comparing, for a number of typical plots, the results obtained by such visual assessment and by application of the method of least squares to the calculation of the slope of the steeper portion of the plot. Of seventeen results examined, only two showed a deviation exceeding 2 %, while the mean deviation was ± 1 %. Thus direct visual assessment, if carefully carried out, is sufficiently accurate for all practical purposes.

According to Meunier [1937] extrapolation to zero time is itself sufficient to eliminate errors due to interference by more slowly reacting non-specific reductants. If this were so, it would be possible, after a suitable calibration of the instrument, to calculate the ascorbic acid content of any mixture from its initial Spekker reading, obtained by extrapolation of experimental readings to zero time; methods of this type have, in fact, already been described [Evelyn *et al.* 1938; Bessey, 1938]. In practice, however, fading of the mixtures is sometimes extremely rapid, and this necessarily means that the accuracy of the extrapolation is correspondingly limited. In fact, as remarked above, the individual results on the steeper portion of the plot frequently deviate somewhat from the theoretical straight line, indicating that these individual results are not free from appreciable errors. The determination of the first appearance of excess indophenol, as indicated by the point of intersection on the plot, is subject to much less uncertainty. In this connexion it may be remarked that during the examination of a large number of materials by the method it was observed that, even when fading of the mixtures was very rapid and extrapolation correspondingly difficult, the shape of the final plot was always quite satisfactory.

EXAMINATION OF THE METHOD

Titration of cysteine and glutathione. Of the various non-specific reductants which may give rise to interference in the titration of ascorbic acid with 2:6-dichlorophenolindophenol, the most common are probably cysteine and glutathione. An examination of the effects of these materials on the estimation of ascorbic acid by the Spekker titration method was therefore made. In the first place titrations were carried out on solutions containing cysteine (in the

Table 1. *Titration of solutions of cysteine (hydrochloride) and glutathione with 2:6-dichlorophenolindophenol*

Amount present* mg./100 ml.	Indophenol required for 4 ml. test solution (ml.)			
	Cysteine (hydrochloride)		Glutathione	
	Visual titration	Spekker photoelectric titration	Visual titration	Spekker photoelectric titration
6·5	0·045 (1 drop)	Nil	0·045	−0·01
26·0	Pink colour after 1 drop, but fugitive in about 20 sec. Similar effect with further additions. Gradual increase in time required to discharge colour, but no true end-point	−0·04	0·045	Nil
65·0	Dye added in 0·2 ml. portions. Pink colour after first addition, but fugitive within 15 sec. Similar behaviour with subsequent additions, complicated by appearance of greenish yellow end-colour. Permanent pink colour at about 12 ml.	−0·04	0·045	Nil

* These figures represent the amount of cysteine present; the amount of hydrochloride taken was appropriately greater.

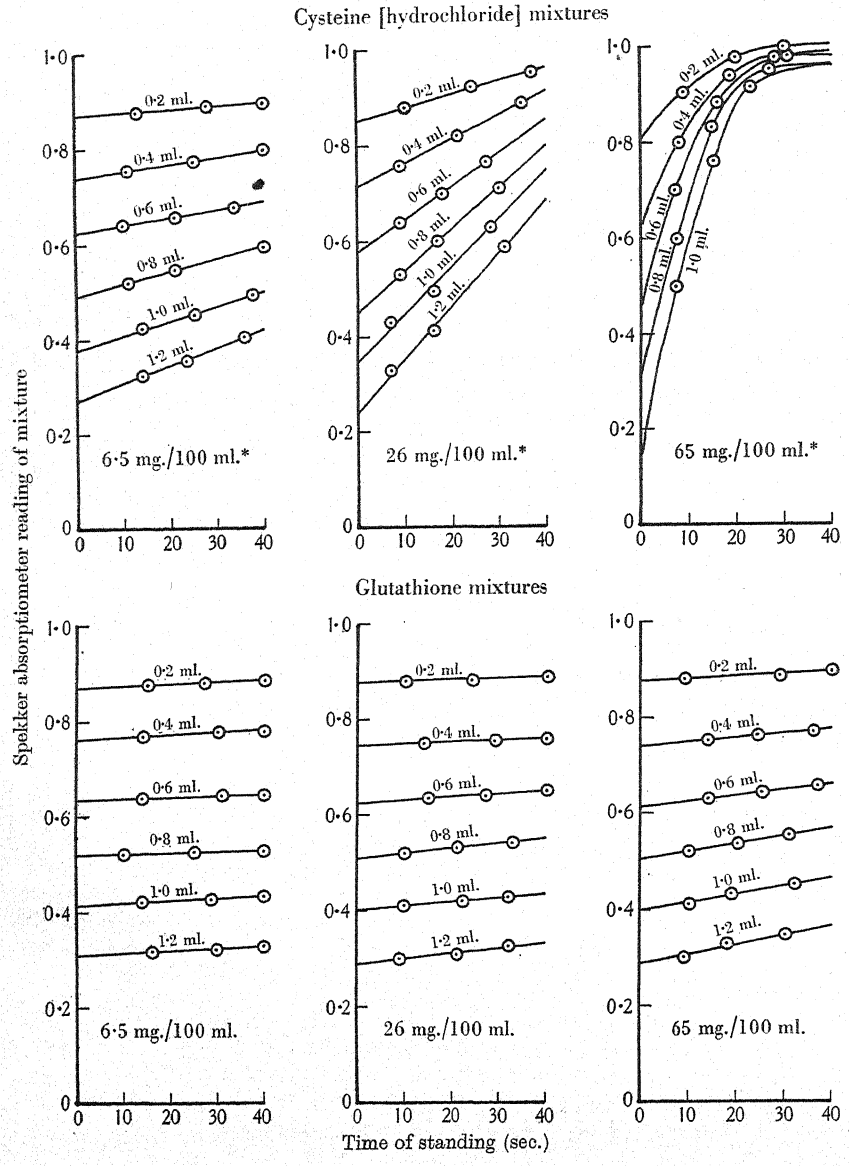

Fig. 1. Fading of solutions containing 2:6-dichlorophenolindophenol in admixture with cysteine (hydrochloride) and glutathione. (Figures attached to curves indicate amount of indophenol solution added to 4 ml. portion of cysteine or glutathione solution.) See note attached to Table 1.

form of its hydrochloride) and glutathione alone. The solutions were prepared in 6 % acetic acid and contained approximately one, five and ten times the amount of ascorbic acid normally aimed at in the test solution; comparative visual titrations with indophenol were made. The results are given in Table 1.

Fig. 1 shows the fading curves of these mixtures; it is to be noted that, since the Spekker absorptiometer measures the amount of residual light trans-

Table 2. *Recovery of ascorbic acid in admixture with cysteine (hydrochloride) and glutathione*

(Paired results for the visual titration were obtained one before and one after the photoelectric titration)

Amount of interfering material present mg./100 ml.*	Ascorbic acid found, mg./100 ml. In presence of cysteine (hydrochloride)		In presence of glutathione	
	Visual titration	Spekker photoelectric titration	Visual titration	Spekker photoelectric titration
Nil	6·28, 6·35 } 6·32	6·10	6·28, 6·35 } 6·32	6·10
6·5	6·40, 6·40 } 6·40	6·16	—	—
26·0	6·52, 6·45 } 6·49	6·10	6·53, 6·40 } 6·47	6·13
65·0	7·8, 7·8 } 7·8†	6·10	6·40, 6·43 } 6·42	6·10

* See note attached to Table 1.

† This figure is very approximate: no definite end-point could be obtained.

It will be observed that even in the absence of any interfering material the visual titration yields higher results than the Spekker photoelectric titration; this is to be expected, since in a visual titration a small excess of indophenol is required to produce a visible colour, whereas the photoelectric procedure should yield the exact end-point of the titration. As the amount of interfering material increases, with its attendant difficulty in the observation of the visual end-point, the apparent recovery of ascorbic acid by the visual titration increases progressively. Recovery by the photoelectric method, apart from slight random variations, remains constant.

The photoelectric titration curves of the mixtures are given in Fig. 2: all the curves are of quite satisfactory shape, although the mixtures containing large amounts of cysteine showed very rapid fading (cf. Fig. 1).

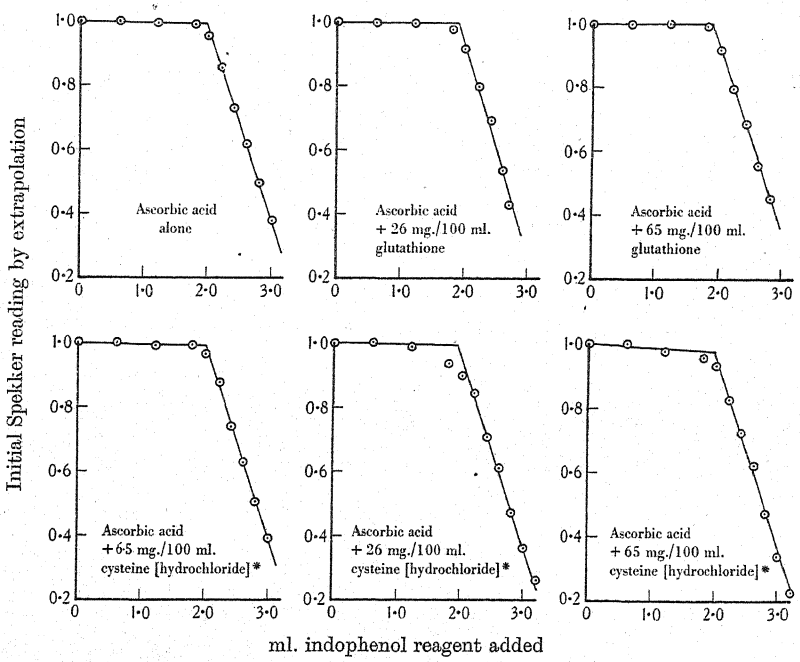

Fig. 2. Photoelectric titration curves of ascorbic acid in admixture with cysteine (hydrochloride) and glutathione. See note attached to Table 1.

mitted, a diminution of colour is indicated by an increase in the instrument reading. In both cases the rapidity of fading depends upon the concentration of each of the reactants; as indicated by the visual observations, however, fading of mixtures containing cysteine is very much more marked than that of mixtures containing corresponding amounts of glutathione; even in greatly excessive concentrations, fading due to the latter material is relatively slow. It is satisfactory that, even with the very rapid fading observed in the presence of large amounts of cysteine, the final plot approached closely to linearity and indicated only a slight negative departure from the theoretical zero titration value.

Recovery of ascorbic acid from mixtures containing cysteine and glutathione. Solutions were prepared in 6% acetic acid containing 6·5 mg./100 ml. of commercial synthetic ascorbic acid in admixture with varying amounts of cysteine (hydrochloride) and glutathione; a control solution was prepared containing ascorbic acid alone. These solutions were titrated with 2:6-dichlorophenolindophenol, both visually and by the Spekker photoelectric method. The results are given in Table 2.

APPLICATION OF THE METHOD TO NATURAL MATERIALS

The method has been applied to the examination of a range of plant materials, including both natural and manufactured products. Some of the materials examined apparently contained appreciable amounts of reducing substance other than ascorbic acid (as shown by the shape of the fading curves in the Spekker titration) and in these cases the Spekker titration gave considerably lower results than a visual titration with indophenol. Table 3 shows some typical results.

The lemon juice showed little variation between the results obtained by the two methods, the small difference observed being attributable to the excess of indophenol required to produce a visible colour. The other materials, however, showed considerable discrepancies and, since there seems no reason to suppose that the Spekker titration could yield low results, it appears that with these materials the visual titration may be considerably in error.

Table 3. *Comparison of visual and Spekker titrations of plant materials with 2:6-dichlorophenolindophenol*

Material	Ascorbic acid found, mg./100 g.	
	Visual titration	Spekker titration
Lemon juice	58·2	55·7
Unripe walnuts, solution in 5% metaphosphoric acid	1628.0	1415·0
Banana honey (concentrated banana juice), solution in 6% acetic acid	Fading prevented definite end-point. First colour corresponded to about 58 mg.; fairly permanent colour corresponded to 70–75 mg.	16·0

SUMMARY

The Spekker photoelectric absorptiometer has been applied to the determination of ascorbic acid in the presence of non-specific reductants by a step-titration method; satisfactory recovery of ascorbic acid was obtained in the presence of cysteine and glutathione in amounts up to ten times that of the ascorbic acid itself.

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Studies on Cholinesterase

1. CHOLINESTERASE AND PSEUDO-CHOLINESTERASE

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The existence of an enzyme capable of hydrolysing acetylcholine into choline and acetic acid, first suggested by Dale [1914], was established by Loewi & Navratil [1926] and has since been confirmed by many investigators.

It is still a matter of controversy whether the hydrolysis of acetylcholine is effected by a non-specific esterase or by a specific enzyme, which acts exclusively on esters of choline. Stedman & Stedman [1935], regarding the enzyme present in blood serum as the specific catalyst for the hydrolysis of choline esters, named it choline esterase. While not insisting on the absolute substrate specificity of the enzyme, Easson & Stedman [1937] maintain that cholinesterase is 'specific to the extent that its principal action is to hydrolyse simple esters of choline'. Vahlquist [1935], on the contrary, denies the specificity of cholinesterase in plasma, claiming that the hydrolysis of acetylcholine is brought about by the same enzyme which hydrolyses tributyrin. The specific nature of the enzyme in tissues, on the other hand, has, apparently, not been questioned, the hydrolytic action of tissue extracts on acetylcholine being indiscriminately referred to as 'cholinesterase activity'.

It is the purpose of this paper to show that there exist in the animal body two esterases capable of hydrolysing acetylcholine: a true cholinesterase acting exclusively on choline esters, and a non-specific enzyme, which hydrolyses not only esters of choline but a variety of non-choline esters as well. Moreover, experiments with both enzymes at high and low concentrations of acetylcholine, have revealed a decisive difference between the two esterases, calling for a sharp distinction of the true cholinesterase from the non-specific enzyme, for which we venture to suggest the name pseudo-cholinesterase.

1. PSEUDO-CHOLINESTERASE

The following three series of experiments, carried out with extensively purified enzyme preparations, are neither consistent with the conception of the specific nature of the 'cholinesterase' present in blood serum, nor do they support the view that the hydrolysis of choline esters by tissues is invariably due to a specific enzyme.

(*a*) *The action of 'cholinesterase' from serum and pancreas on non-choline esters**

Horse serum and crude tissue extracts from pancreas (dog) hydrolyse not only esters of choline, but also have a marked hydrolytic effect on non-choline esters such as tributyrin, tripropionin and methyl butyrate. Moderate purification of the 'cholinesterase' from these sources results in a considerable loss of activity towards non-choline esters; but once a hundred-fold increase in 'cholinesterase' activity has been attained, the ratio $Q_{Ach}/Q_{non\text{-}ChE}$ remains constant upon further purification. Even when the activity of the 'cholinesterase' has been increased about two thousand times this ratio is still the same (Table 1).

A variety of steps is required in order to proceed from a hundred-fold to a two thousand-fold increase in 'cholinesterase' activity. If we assume that two distinct enzymes are present in our hundred-fold purified extracts, the one acting on acetylcholine, the other on non-choline esters, it seems reasonable to expect that the ratio $Q_{Ach}/Q_{non\text{-}ChE}$ would change during the process of further purification. The fact that this ratio remains constant suggests that a single enzyme is responsible for the observed choline and non-choline ester hydrolysis. The following experiments verify this hypothesis.

* The method for the purification of the tissue 'cholinesterase' (dog pancreas) used in our experiments will be found in this issue [Mendel & Mundell, 1943]; the purification of the pseudo-cholinesterase from horse serum will be published later.

The activity of the enzyme preparation was measured manometrically by Warburg's method at 37·5° in 0·025 *M* $NaHCO_3$ saturated with 5% CO_2 (*p*H 7·4). Each molecule of acid liberated from choline esters or non-choline esters during hydrolysis, releases one molecule of CO_2 from the bicarbonate. Therefore, the quotient

$$\frac{[\text{volume of } CO_2 \text{ (in } \mu\text{l.)}]}{[\text{time (in hr.)}] \times [\text{dry-wt. of enzyme preparation (in mg.)}]},$$

which will be denoted by the symbol Q, is a measure of the activity of the enzyme preparation. The suffix to Q refers to the substrate: Ach = acetylcholine, Tb = tributyrin, Mb = methyl butyrate, Tp = tripropionin and non-ChE = non-choline esters.

Table 1. *Hydrolysis of choline and non-choline esters by 'cholinesterase' preparations of varying purity*

Substrate concentration 0·03 *M*

Source of 'cholinesterase'	Increase in 'cholinesterase' activity	Q_{Ach}*	Q_{Tb}†	Q_{Mb}	Q_{Tp}	$\frac{Q_{Ach}}{Q_{Tb}}$	$\frac{Q_{Ach}}{Q_{Mb}}$	$\frac{Q_{Ach}}{Q_{Tp}}$
Horse serum	0	34	60	30	—	0·57	1·13	—
,,	118-fold	4,000	1,280	90	—	3·12	44·5	—
,,	472-fold	16,000	5,400	—	—	2·96	—	—
,,	1670-fold	57,000	19,000	1,250	—	3·00	45·5	—
Dog pancreas	0	310	15,500	—	—	0·02	—	—
,,	97-fold	30,000	9,100	520	7,200	3·29	57·5	4·15
,,	1930-fold	600,000	181,000	10,800	150,000	3·31	55·5	4·00

* Acetylcholine chloride (Hoffmann-La Roche) was used in all experiments.

† Since the tributyrin at our disposal contained some inhibitory substance, it was purified by fractionated distillation in a high vacuum (0·003 mm. Hg). The pure substance distilled at b.p. 97°–98° (bath temperature 135–140°), $n_D^{26°} = 1{\cdot}4340$.

(*b*) *The effect of eserine on choline and non-choline ester hydrolysis*

Easson & Stedman [1937], experimenting with various blood sera, found that about a thousand times the amount of eserine sufficient for the complete inhibition of choline ester hydrolysis only partially abolished the hydrolysis of non-choline esters.

We are able to confirm these observations, and agree with Stedman's interpretation that a special enzyme, esterase, capable of hydrolysing non-choline esters and scarcely sensitive to eserine, is present in certain untreated sera. As such sera (guinea-pig, horse) and crude tissue extracts (dog pancreas) contain far greater quantities of esterase than of 'cholinesterase' the action of the former would obviously conceal the inhibitory effect of eserine on the activity of the latter towards non-choline esters.

Table 2. *The effect of eserine on the hydrolysis of acetylcholine and tributyrin by purified 'cholinesterase' preparations*

Source of 'cholinesterase' preparation	Eserine concentration *M*	Hydrolysis (in μl. CO_2 evolved in a 5 min. period) of: Acetylcholine (0·003 *M*)	Tributyrin (0·003 *M*)	% inhibition of hydrolysis of: Acetylcholine	Tributyrin
Horse serum	0	66·0	22·5	—	—
(Q_{Ach}16,000)	10^{-8}	42·5	15·5	36	31
	10^{-7}	9·0	2·5	86	89
	10^{-6}	0	0	100	100
Dog pancreas	0	39·5	12·5	—	—
(Q_{Ach} 600,000)	10^{-8}	20·5	6·0	48	52
	10^{-7}	7·0	2·5	82	80
	10^{-6}	3·5	1·5	91	88

We decided, therefore, to examine the effect of eserine on the non-choline ester hydrolysis brought about by our purified extracts, from which we believed the esterase had been removed (see section 1, *a*).

Table 2 shows that small amounts of eserine inhibit the hydrolysis of acetylcholine and of non-choline esters to practically the same extent. Apart from confirming the absence of esterase, these experiments make it highly improbable that two enzymes—the one acting on choline esters, the other on non-choline esters—are present in our purified preparations, thus supporting our hypothesis that the 'cholinesterase' in serum and pancreas is a non-specific enzyme.

(*c*) *The action of serum and tissue extracts on mixed substrates*

If tissue extracts or sera contained two enzymes capable of hydrolysing substrates A and B respectively, addition of the activities of both enzymes should occur in the presence of the two substrates, provided the substrate concentrations were optimal and substrate A did not inhibit the hydrolysis of substrate B and vice versa. The latter point has already been dealt with by Stedman [1935], who found that choline esters do not inhibit appreciably, if at all, the hydrolysis of tributyrin. Experimenting with various blood sera, Easson & Stedman [1937] observed that addition of activities did occur in the presence of two substrates (tributyrin and butyryl choline), the addition being almost complete in the case of guinea-pig serum, partial in the case of horse serum and less pronounced in the case of human

serum. Once more we are able to confirm these results for untreated sera and crude tissue extracts, and we again agree with Stedman's interpretation that the addition of activities is caused by a second enzyme, esterase, abundant in the sera of guinea-pig and horse.

If, however, purified preparations from which the esterase has been removed (see Section 1, *b*) are allowed to act on a mixture of two substrates, a choline ester (acetylcholine) and a non-choline ester (tributyrin), no additive effect can be observed (Table 3).

Table 3. *The hydrolysis of mixed substrates by purified 'cholinesterase' preparations*

Source of 'cholinesterase' preparation	μl. CO_2 evolved in 5 min. from Acetylcholine (0·03 *M*)	Tributyrin (0·03 *M*)	Acetylcholine (0·03 *M*) + tributyrin (0·03 *M*)
Serum (Q_{Ach} 16,000)	105	34	100
Pancreas (Q_{Ach} 600,000)	42	13	42

This result is consistent with our assumption that the hydrolysis of choline esters in serum and certain tissues is brought about by an enzyme also capable of hydrolysing non-choline esters.

We have presented and discussed three types of evidence in support of our hypothesis that one enzyme is the agent for the hydrolysis of choline and non-choline esters in our purified 'cholinesterase' preparations.

(*a*) the unaltered $Q_{Ach}/Q_{non\text{-}ChE}$ ratio within the range of hundred-fold to two thousand-fold purification;

(*b*) the identical inhibitory effect of eserine on choline ester and non-choline ester hydrolysis, when 'cholinesterase' alone is present;

(*c*) the complete lack of addition of activities when a choline and a non-choline ester are hydrolysed together.

The concurrence of these three different lines of evidence can, we believe, be accepted as conclusive proof that the 'cholinesterase' in horse serum and dog pancreas is a non-specific enzyme.

The term cholinesterase, suggestive of substrate specificity, should, therefore, not be applied to the non-specific enzymes in sera and certain tissues and might be replaced by the term pseudo-cholinesterase.

2. THE SPECIFIC OR TRUE CHOLINESTERASE

Extending our studies on the specificity of cholinesterase to other mammalian tissues, we discovered in red blood cells and brain tissue of various species a true cholinesterase, acting exclusively on choline esters.

Cholinesterase in red blood cells. Table 4 shows that laked red blood cells are capable of hydrolysing both acetylcholine and non-choline esters, such as tributyrin and methyl butyrate. By a simple method of purification (see Appendix) blood cell extracts were obtained, displaying increased activity per unit weight towards acetylcholine, while being completely inactive towards tributyrin and methyl butyrate (Table 4).

Table 4. *The activity, before and after purification, of cholinesterase in red blood cells towards choline and non-choline esters*

Source of red blood cells	Q_{Ach} Before purification	Q_{Ach} After purification	Q_{Tb} Before purification	Q_{Tb} After purification	Q_{Mb} Before purification	Q_{Mb} After purification
Human	23·0	440	5·4	0	2·5	0
Sheep	11·5	183	6·5	0	2·0	0
Horse	7·0	178	2·6	0	—	—
Ox	19·5	340	3·0	0	—	—

Substrate concentration 0·003 *M*.

These experiments, we believe, justify the conclusion that a specific cholinesterase is present in the red blood cells of various mammals.

Cholinesterase in brain. Brain tissue (mouse, dog), which effectively hydrolyses acetylcholine, exhibits some activity towards tributyrin and tripropionin, but apparently none towards methyl butyrate (Table 5). Unfortunately, brain cholinesterase does not lend itself readily to purification, and has not yet been separated from the other esterases present in brain tissue.

Nevertheless, the effect of eserine on the choline and non-choline ester hydrolysis, brought about by brain tissue, and the action of brain tissue on mixed substrates, enable us to arrive at a definite conclusion regarding the specificity of the cholinesterase in brain. Similar experiments with certain other unpurified esterase preparations, e.g. guinea-pig serum or crude pancreas extracts, would have been inconclusive, since the hydrolysis of non-choline esters by these preparations, far exceeding the hydrolysis of acetylcholine, would have concealed the presence of pseudo-cholinesterase (see Section 1, *b*). No such interference, however, occurs in experiments with crude brain preparations, owing to the fact that brain tissue, with rare exceptions, hydrolyses choline esters far more readily than non-choline esters.

(*a*) *The effect of eserine on choline and non-choline ester hydrolysis brought about by brain tissue*

Table 5 shows that an amount of eserine which almost completely inhibits the hydrolysis of acetylcholine has practically no effect on the hydrolysis of tributyrin. Indeed, the concentration of eserine must be increased more than hundred-fold before any appreciable inhibition is observed.

Table 5. *Effect of eserine on the hydrolysis of choline and non-choline esters by cholinesterase in brain*

The brain tissue was ground and suspended in about 5 volumes of distilled water, centrifuged, and the precipitate containing the cholinesterase was again suspended in the same volume of water. 0·2 ml. of this suspension was employed in all experiments except the last set (Nucleus caudatus) where 0·05 ml. was used).

Source of cholinesterase	Molar concentration of eserine	μl. CO_2 evolved in 10 min. from Acetylcholine	Tributyrin	Methylbutyrate	Tripropionin
Mouse brain (whole)	0	53·0	34·0	0	—
	10^{-6}	7·0	34·0	—	—
	10^{-5}	0	34·0	—	—
	10^{-4}	0	36·0	—	—
Dog brain (grey matter, cortex)	0	27·5	34·5	0	—
	10^{-6}	0	—	—	—
	10^{-5}	0	33·0	—	—
	10^{-4}	0	35·0	—	—
Dog brain (white matter)	0	62·0	26·0	—	—
	10^{-6}	4·0	24·5	—	—
	10^{-5}	0	23·5	—	—
	10^{-4}	0	20·0	—	—
Dog brain (grey matter, Nucleus caudatus)	0	92·0	—	—	9·0
	10^{-7}	8·0	—	—	8·0
	10^{-6}	0	—	—	8·0

If one enzyme were responsible for the hydrolysis of acetylcholine and tributyrin—as was the case in experiments with purified enzyme preparations from serum and pancreas (Table 2)—small amounts of eserine would inhibit the hydrolysis of both esters to the same extent. The fact that a concentration of eserine, which completely inhibits the hydrolysis of acetylcholine, has little or no effect on the hydrolysis of tributyrin, shows that the enzyme responsible for the hydrolysis of acetylcholine has practically no share in the hydrolysis of non-choline esters and can, therefore, be regarded as a specific cholinesterase.

(*b*) *The action of brain tissue on mixed substrates*

Table 6 gives the results of two typical experiments in which a choline ester (acetylcholine) and a non-choline ester (tributyrin) are added to a suspension of ground brain tissue both separately and together. When the two substrates are added together, the hydrolytic action of the brain tissue is exactly equal to the sum of its separate actions on the choline and the non-choline ester respectively.

If a pseudo-cholinesterase, i.e. an enzyme capable of hydrolysing both substrates, were present, the activity of the brain tissue towards the substrate mixture would be less than the sum of the activities which were observed when the substrates were added separately.

The fact that complete addition of activities takes place, reveals the presence of at least two distinct ester-hydrolysing enzymes, and excludes the existence of a pseudo-cholinesterase in brain tissue.

Table 6. *The hydrolysis of mixed substrates by suspensions of ground brain tissue*

The suspensions were prepared as described in Table 5.

Cholinesterase preparation	μl. CO_2 evolved in 5 min. from Acetylcholine (0·003 *M*)	Tributyrin (0·003 *M*)	Acetylcholine (0·003 *M*) + tributyrin (0·003 *M*)
Dog brain (grey matter, nucleus caudatus)	68	6	74
Mouse brain (whole)	36	12	48

The above experiments prove, we believe, that the cholinesterase in the brain of mammals is a specific enzyme.

The true cholinesterase, found in brain tissue and red blood cells, may also be present in other tissues not yet investigated. Even in tissues such as pancreas, in which, according to our experiments, the hydrolysis of acetylcholine seems to be entirely due to the action of a pseudo-cholinesterase, the presence of small amounts of true cholinesterase cannot be excluded.

3. THE ACTIVITY OF CHOLINESTERASE AND PSEUDO-CHOLINESTERASE AT VARIOUS CONCENTRATIONS OF ACETYLCHOLINE

The pseudo-cholinesterases in human and horse serum exhibit their maximum activities at acetylcholine concentrations of about 1·0 and 0·3 % respectively [Glick, 1937; Stedman, 1935], concentrations far higher than could be expected to occur in the blood stream. At relatively low concentrations of acetylcholine, still too high, however, to be compatible with life, the pseudo-cholinesterases display only a fraction of their maximum activity. If, for instance, 300 mg. of acetylcholine are added to 100 ml. of horse serum (38°, *p*H 7·4), we find that the hydrolysis of 3 mg. of the substrate is a matter of no more than 20 sec.; if, on the other hand, only 3 mg. of acetylcholine are added to 100 ml. of serum, 3 min. are required for the hydrolysis of the same amount of substrate.*

Unlike pseudo-cholinesterase, the true cholinesterase, found in brain tissue and red blood cells, is far more effective at low concentrations of acetylcholine; indeed, the lower the substrate concentration, the greater the activity of the specific cholinesterase (Tables 7 and 8). It may be noted that recently, Alles & Hawes [1940], continuing the work of Galehr & Plattner [1928], made the interesting observation that human red blood cells,

* The activity-substrate concentration curve of the pseudo-cholinesterase from dog pancreas is almost identical with the curve of the pseudo-cholinesterase from horse serum.

which according to our experiments contain the specific cholinesterase, were responsible for most of the action of whole blood on acetylcholine, when the substrate was present at low concentrations. They also found that high concentrations of acetylcholine inhibited the action of cholinesterase in human red blood cells. At the same time they noted differences between the serum cholinesterase, which we have found to be a pseudo-cholinesterase, and the cholinesterase present in red blood cells.

Table 7. *Hydrolysis of acetylcholine by cholinesterase from red blood cells*

The horse blood cells were purified in the manner described in the Appendix.

Concentration of acetylcholine mg. %	μl. CO_2 evolved in the 2 min. period following addition of substrates to haemolysed blood cells of			
	Man	Sheep	Horse	Ox
500·0	10·0	13·5	6·0	12·5
50·0	17·0	25·0	19·5	—
25·0	—	30·0	20·5	28·0
2·5	19·0	—	24·0	32·0

Table 8. *Hydrolysis of acetylcholine by cholinesterase in brain*

The brain suspensions were prepared as described in Table 5.

Concentration of acetylcholine mg. %	μl. CO_2 evolved in the 2 min. period following addition of substrates to suspensions of		
	Mouse brain (whole)	Dog brain (grey matter, cortex)	Dog brain (whole)
500·0	5·0	4·5	4·0
50·0	—	11·0	8·0
25·0	13·0	—	11·0
12·5	19·0	14·0	—
5·0	21·0	16·5	—
2·5	22·5	18·5	—

Since it seems reasonable to assume that usually low concentrations of acetylcholine occur in living organisms, it is most likely that the destruction of acetylcholine, wherever it takes place rapidly, is brought about mainly, if not exclusively, by the specific cholinesterase.

SUMMARY

Experiments performed with highly purified esterase preparations capable of hydrolysing acetylcholine, show that the 'cholinesterase' present in blood serum and certain tissues is a non-specific enzyme, catalysing the hydrolysis not only of choline esters but of non-choline esters as well.

A specific cholinesterase, however, acting exclusively on choline esters, was found in the brain tissue and the red blood cells of some mammals.

The non-specific 'cholinesterase', purified from horse serum and dog pancreas, exhibits its maximum activity at high concentrations of acetylcholine (above 300 mg./100 ml.) and only slight activity at low substrate concentrations. The reverse is true for the specific cholinesterase, found in brain tissue and red blood cells: while inhibited by high concentrations of acetylcholine, it displays its maximum activity at low substrate concentrations (below 3 mg./100 ml.).

It is proposed to name the non-specific enzyme pseudo-cholinesterase, and to reserve the term cholinesterase for the specific enzyme.

APPENDIX

Method for the separation of cholinesterase from other esterases in red blood cells

10–15 ml. of freshly drawn oxalated blood (human, horse, sheep, ox) are centrifuged, and the plasma is discarded. The red blood cells are washed twice with about 30 ml. of physiological saline solution. A layer of white blood cells which forms on top of the red blood cells after centrifuging, is removed as completely as possible, by suction with a capillary.

To 5 ml. of the washed red blood cells 45 ml. of distilled water are added. When complete haemolysis has taken place, the solution is shaken thoroughly with 1 g. of kieselguhr (B.D.H., acid-washed). The mixture, including all the kieselguhr, is then filtered through filter paper on a Büchner funnel, 7 cm. in diameter. The filtrate, containing practically all the haemoglobin, is poured back, until it is absolutely clear, and is then discarded. The almost-dry filter cake is scraped from the filter paper, thoroughly shaken with 50 ml. of distilled water and the filtration repeated as previously described.

The filter cake is again scraped from the filter paper and suspended in 5 ml. $N/1000$ NaOH. The suspension is shaken thoroughly for a few minutes and then centrifuged at high speed.

The slightly turbid supernatant fluid, orange-red in colour, is 20–30 times as active (per unit dry weight) as the original blood cells towards acetylcholine, and inactive towards non-choline esters, such as tributyrin.

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Studies on Cholinesterase

2. A METHOD FOR THE PURIFICATION OF A PSEUDO-CHOLINESTERASE FROM DOG PANCREAS

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Many papers have been published on the properties and distribution of cholinesterase in the animal body but few attempts have been made at its purification. Stedman & Stedman [1935] purified the enzyme from horse serum 50 to 100 times; McMeekin [1939], using a different method, slightly increased the degree of purity. In this laboratory preparations about 20 times as active as Stedman's were procured from horse serum, but the yield was very low and the method not suitable for operations on a larger scale.

The only previous attempt to purify cholinesterase from tissues was made by Nachmansohn & Lederer [1939], who obtained aqueous extracts from the electric organ of the *Torpedo vulgaris* which were about three times as active as Stedman's purest preparations.

For the purification of tissue cholinesterase, to be described in this paper, dog pancreas was chosen, as it was known to contain large amounts of the enzyme [Marnay, 1938]. The pancreas of larger animals such as horse, ox and hog were tested for cholinesterase activity, but were so much less active than dog pancreas that purification was not attempted.

The method of purification is relatively simple. Only two chemicals, $(NH_4)_2SO_4$ and kieselguhr, are used in the process, which consists of four main steps: (1) extraction of the enzyme with water; (2) fractionation with $(NH_4)_2SO_4$; (3) adsorption on to kieselguhr, and (4) elution with ammonium sulphate.

The final preparation, 1 mg. of which hydrolyses about 1·2 mg. of acetylcholine per sec., is approximately 2000 times as active per unit dry weight as its source and 100–200 times as active as the purest preparations described by McMeekin or Stedman.

The purified enzyme proved to be a pseudo-cholinesterase [Mendel & Rudney, 1943], since it attacked a variety of non-choline esters and hydrolysed acetylcholine with maximum speed at a comparatively high substrate concentration (about 0·02 *M*).

EXPERIMENTAL

Estimation of cholinesterase activity

The activity of the enzyme is measured manometrically by the Warburg method, first adopted for the measurement of cholinesterase activity by Ammon [1933]. An aqueous solution of the enzyme preparation (0·1 ml. or less) is placed in the main compartment of a Warburg flask and brought to a volume of 1·9 ml. with a solution of 0·025 *M* $NaHCO_3$, previously saturated with 5 % CO_2 in N_2. The flask is attached to the manometer and filled with the above-mentioned gas mixture, in order to maintain the enzyme solution at a constant *p*H (7·4). 0·1 ml. of a 10 % aqueous solution of acetylcholine chloride (Hoffmann la Roche) is tipped from the side bulb into the main compartment after temperature equilibrium at 37·5° has been attained.

1 m.-mol. of acetylcholine, when hydrolysed by the enzyme into choline and acetic acid, evolves 1 m.-mol. of CO_2 from the $NaHCO_3$. The amount of CO_2 in μl. evolved in 1 hr. by 1 mg. dry weight of the enzyme preparation, is referred to as the acetylcholine quotient (Q_{ACh}).

METHOD OF PURIFICATION

1. *Extraction*

The pancreas is removed from 4 or 5 dogs immediately after electrocution, rinsed with cold water, and freed from fat, mesentery and visible blood vessels. The tissue, weighing about 100 g., is cut into small pieces, finely minced (an electric mixer, 'Whizmix', was found very efficient for mincing and mixing the tissue with water) and extracted with 9 parts of cold distilled water. Extraction is allowed to continue for at least 5, but not longer than 20 hr., in the refrigerator (about +2°); the mixture is then strained through a coarse sieve (18 mesh) to remove fibrous tissue. The Q_{ACh} of the strained suspension varies from 200 to 400, the higher values generally being obtained during July, August and September.

2. *Fractionation with ammonium sulphate*

A. *Precipitation at 0·48 saturation.* The strained suspension is gradually brought to 0·48 saturation with an $(NH_4)_2SO_4$ solution (sat. at 20°) by adding 2/3 of the required $(NH_4)_2SO_4$ at once and the remainder 2 hr. later: this prevents any of the albumin fraction from being carried down prematurely with the bulky precipitate. Throughout this procedure, and for 3 or 4 hr. afterwards—the time required for

complete flocculation—the mixture is allowed to stand in the refrigerator. At this stage of purity the enzyme preparation can be kept in the cold for weeks without loss of activity.

The flocculent suspension is then centrifuged in a high speed centrifuge (a Sharples Super Centrifuge run by air pressure) and the precipitate is discarded.* The active (0·48 sat.) fluid is brought to 0·85 sat. with solid $(NH_4)_2SO_4$ in order to concentrate the enzyme by precipitation. The mixture, having been kept in the cold for at least 3 hr., is repeatedly filtered through filter paper on a Büchner funnel by suction, until the filtrate is clear.

The active filter cake is scraped from the filter paper and thoroughly mixed with a quantity of water equal to 1/20–1/10 of the original volume of the extract. The mixture is centrifuged to eliminate coarse insoluble material; a floating layer of fat, which may have formed, is removed as completely as possible. The turbid supernatant fluid is then dialysed in cellophane bags against running tap water (below 15°) for at least 16 but not longer than 20 hr. An inactive precipitate which forms during dialysis is removed by centrifuging.

The dialysed enzyme solution is again brought to 0·85 sat. with solid $(NH_4)_2SO_4$ and filtered as described above. The filter cake is dissolved in the smallest possible amount of water and dialysed against running tap water for 20 hr. After dialysis any precipitate is centrifuged off and the brownish coloured enzyme solution is dried in vacuo over $CaCl_2$ at a temperature not exceeding +2°. At this stage the Q_{ACh} ranges from 8000 to 12,000. Since the dried active material is stable when kept on ice, it can conveniently be collected and stored at this point.

When a sufficient quantity of the dried preparation (3–4 g.) has been accumulated, it is dissolved in about 10 parts of cold distilled water. Much insoluble inactive material is removed by centrifuging, and the enzyme solution is dialysed in the refrigerator for 36 hr. against frequently changed distilled water. After dialysis the Q_{ACh} is about 20,000.

B. *Precipitation at 0·5 saturation.* An equal volume of sat. $(NH_4)_2SO_4$ is added to the dialysed enzyme preparation, and the mixture is kept in the refrigerator for about 1 hr. The resulting flocculent precipitate is inactive and should be removed by centrifuging.

C. *Precipitation at 0·52 saturation.* By addition of more saturated $(NH_4)_2SO_4$ solution, the active 0·5 sat. fluid is brought to exactly 0·52 sat. and is then kept at 37° for 15 min. An inactive precipitate forms and is removed by centrifuging. The active fluid is again concentrated by precipitation with solid $(NH_4)_2SO_4$ (0·85 sat.) and is filtered as before. The filter cake is dissolved in as small a volume of water as possible and dialysed against running water (below 15°) for 20 hr. At this stage the preparation has a Q_{ACh} of approximately 40,000. It may be dried in vacuo at a temperature not exceeding 2° and stored in the cold for about 2 months without loss of activity.

3. *Adsorption on to kieselguhr*

When at least 0·5 g. of the dried enzyme material with a Q_{ACh} of about 40,000 has been accumulated, it is dissolved in about 20 parts of cold water and dialysed against frequently changed distilled water in the refrigerator for 36 hr. After dialysis, the brownish coloured liquid is shaken for 10 min. with the least amount of kieselguhr* which will adsorb about 95 % of the active material. The kieselguhr suspension is cooled in ice-water and centrifuged for a few minutes. The supernatant liquid, containing the greater part of the brownish coloured inactive material, is discarded. The kieselguhr is washed with cold distilled water and then centrifuged; this is repeated several times until the water has become colourless. Very little active material is lost in the course of this procedure.

4. *Elution*

A cold 0·1 sat. $(NH_4)_2SO_4$ solution, equal in volume to the enzyme solution before adsorption, is added to the kieselguhr. The suspension, while being cooled in ice-water, is stirred mechanically by a glass rod for 10–15 min. It is then centrifuged for a very short time. The resulting supernatant liquid contains 40–60 % of the adsorbed active material and the yield can be increased to about 75 % by a second elution.

The combined clear eluates are brought to 0·85 sat. with solid $(NH_4)_2SO_4$ and are kept in the cold for at least 6 hr. A flocculent precipitate forms and the suspension is filtered repeatedly through filter paper on a small Büchner funnel (diameter *ca.* 2 cm.) by suction until the filtrate is perfectly clear and the filter cake almost dry. The filter paper with the adhering layer of precipitate is placed in a small beaker containing not more than 5 ml. of ice-cold distilled water. Unless a fairly concentrated solution of the enzyme material is prepared, considerable loss of activity may occur during dialysis. The active material, which dissolves readily, is transferred to a cooled centrifuge tube, and the liquid absorbed by the filter paper is squeezed out and added. The

* The esterase is associated with both the globulin and albumin fractions of the pancreas proteins, but since it was found very difficult to purify the globulin part, only the albumin fraction (containing about 1/2 of the esterase activity) was used for further purification.

* About 3 g. of kieselguhr 'Brocades-Stheeman (Amsterdam)' are required for 50 ml. of the enzyme solution. Other brands of kieselguhr, such as B.D.H. kieselguhr, white, are suitable but not quite as efficient.

solution, having been cooled, is centrifuged for 2 or 3 min. to remove any traces of filter paper, and should then be dialysed for 2 days in the refrigerator against frequently changed distilled water. An inactive precipitate may form which can be eliminated by centrifuging. The enzyme solution is now colourless and has a Q_{ACh} of 400,000–600,000.

The yield of this purified preparation amounts to about 20 % of the original activity of the pancreas, but is much greater (40 %) when calculated on the basis that only the albumin fraction is used for purification.

Since enzyme preparations with a Q_{ACh} above 10,000 are adversely affected by salts and dilution with water,* they should be diluted, and their activity measured, in a solution of 0·25 % gum acacia.

* Effects of salts and dilution with water will be discussed in a subsequent paper dealing with the properties of the purified enzyme.

SUMMARY

A simple method is described by which a tissue esterase, capable of hydrolysing acetylcholine, may be purified from dog pancreas. The final preparation, 1 mg. of which hydrolyses about 70 mg. of acetylcholine per minute, is 2000 times as active per unit dry weight as the original material and about 15,000 times as active as horse serum.

The enzyme is a pseudo-cholinesterase, since it hydrolyses esters other than those of choline, and splits acetylcholine with maximum speed at a substrate concentration (0·02 *M*) far above physiological range.

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Methods for the Purification of Tomato Bushy Stunt and Tobacco Mosaic Viruses

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Since we first described methods for making liquid crystalline preparations of tobacco mosaic virus [Bawden & Pirie, 1937] and crystalline preparations of tomato bushy stunt virus [Bawden & Pirie, 1938], we have made preparations of both viruses by many other methods. As a result, we can now describe simpler and better procedures. Other workers have used differential ultracentrifugation; because of the infrequency with which stable substances of high molecular weight seem to occur in normal leaves, this method of preparation is undoubtedly of great value. The handling of large volumes of sap in the ultracentrifuge, however, is laborious, and many laboratories where plant viruses are studied do not have this equipment. Methods of preparation that are trustworthy and involve only ordinary centrifuges may therefore be of more general use.

Tomato bushy stunt virus

In our earlier method the sap was heated to 60° to coagulate the normal leaf protein, and if the preparation is needed solely for a study of its serological or physical properties this is still probably the simplest procedure. However, heating to this extent greatly reduces the infectivity of the virus [Smith, 1935; Stanley, 1940], apparently without affecting its other properties appreciably, and more infective preparations can be made in other ways. The method described below is the most satisfactory we have found, but with small quantities of sap, precipitation with alcohol, as used by Pirie, Smith, Spooner & McClement [1938] for tobacco necrosis viruses, is also suitable and quicker.

Preparation. Leaves and stems of infected tomato plants are put through a domestic meat mincer with $\frac{3}{16}$ in. holes in the plate, and sap is expressed from the mince by hand through a bag of thin, closely woven cloth such as madapollam. For each 100 ml. of sap expressed, 30 ml. of a 4 % solution of anhyd. Na_2HPO_4 is added to the residue in the bag; when this has soaked in, the residue is again put through the mincer and the sap is expressed as before. The two extracts are mixed, centrifuged and the precipitate discarded. The supernatant fluid is usually clear and brown,

but it sometimes has a greenish tinge; this can be removed by adding some more phosphate solution. $(NH_4)_2SO_4$ is added (280 g./l.), and after it has all dissolved the fluid is left for about 12 hr. at room temperature. The precipitate is then centrifuged off, and suspended evenly in a volume of water equal to about one-tenth of the original sap. The fluid is then centrifuged and the precipitate extracted twice or thrice more with smaller volumes of water. The three supernatant fluids are mixed, acetic acid is added to bring the *p*H to 4, the bulky precipitate is centrifuged off, and extracted twice with water. All subsequent operations are carried out at *p*H values between 4 and 4·5.

$(NH_4)_2SO_4$ is added to the mixed acid fluids until there is a definite turbidity; it is convenient to add the solid until about 15 g./100 ml. have been added and then to continue with a saturated solution. Within a few hours at room temperature a precipitate separates from the turbid solution. This can be centrifuged off after about 6 hr. and contains most of the virus. After standing for a few days, however, or after the addition of a little more salt, a further precipitate containing a little virus may separate from the supernatant fluid. This is most conveniently worked up separately. The precipitate is extracted two or three times with dilute acetate buffer so that the volume of the combined extracts is about one-hundredth of that of the original sap. The extract should be colourless or light brown, and only slightly opalescent. At this stage, if old plants have been used, the fluid may still be dark brown; it should then be reprecipitated with $(NH_4)_2SO_4$ and the precipitate, suspended in the minimum amount of water, should be dialysed. As dialysis proceeds a heavy brown precipitate separates; this is centrifuged off, washed once and the fluid used for the extraction of precipitates obtained at a later stage. Saturated $(NH_4)_2SO_4$ solution is now added drop by drop with thorough mixing at a temperature between 20 and 25° until the fluid becomes permanently turbid. Solutions containing more than 1% of virus will begin to crystallize when the $(NH_4)_2SO_4$ concentration has been raised to 10%, but 15% will be needed if the virus concentration is as low as 1 g./l. A precipitate separates after 1–2 min. but dissolves again almost completely when the mixture is cooled to 0°. After 2–3 hr. at 0° any material that remains undissolved is removed by centrifuging at 0°. This can be done in a refrigerated centrifuge or by putting the centrifuge inside a refrigerator. It can, however, conveniently be done in an ordinary machine at room temperature by use of the device illustrated in Fig. 1. A wooden block and lid are turned to fit easily inside the 250 ml. bronze bucket of an International centrifuge and a hole is bored in the block to contain a 50 ml. glass or metal centrifuge tube. If the upper surface of the lid lies a few mm. below the rim of the bronze bucket there is no risk of its being blown off when the centrifuge is spinning. The virus from 1 l. of sap can conveniently be held in four $3 \times \frac{3}{8}$ in. test-tubes, and these are carried, surrounded by ice and water, in the 50 ml. tube. In the model that we use the wooden walls and lid are 12 mm. thick, and some ice remains unmelted after 45 min. centrifuging at 2000 r.p.m. The precipitate can generally be packed tightly by 15 min. centrifuging; if so much $(NH_4)_2SO_4$ has been added that an appreciable amount of virus has also precipitated, it is well to extract the precipitate with water and repeat the precipitation at 0° on the extract.

At this stage the supernatant fluid should be clear and colourless; when left at 0° crystallization will start in a few hours, but if it is allowed to warm up there will be rapid separation of an amorphous product. More virus can be separated in the first fraction if, after a few days, another drop of saturated $(NH_4)_2SO_4$ solution is added for each ml. of fluid. Most of the crystals form on the walls of the tube and, after 4–7 days, the mother liquor can be poured off without centrifuging. If the addition of $(NH_4)_2SO_4$ has been gauged accurately only a tenth of the virus will remain in the mother liquor; this precipitates when the fluid is allowed to warm up and can be centrifuged off, redissolved and crystallized. The crystals do not dissolve completely unless they are left in water for about an hour,

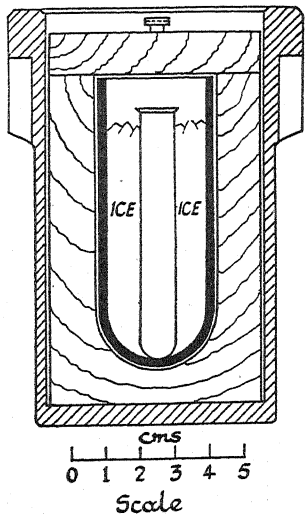

Fig. 1.

and a useful separation from residual contaminants can be effected by washing them quickly. When they have dissolved, any insoluble material is removed, by centrifuging, either immediately or after dialysis. Crystallization seems to proceed equally satisfactorily at any *p*H between 3 and 7, but the removal of traces of denatured virus and normal leaf protein is easiest at about *p*H 4·5.

One variant of this method may on occasion be useful. The addition of enough acid to bring the *p*H of the original sap to 4·5 removes more normal protein than the addition of phosphate and leads to no greater loss of virus. It does not, however, remove so much of the Ca from the sap, and this, if not removed, precipitates with the $(NH_4)_2SO_4$ and may interfere with the subsequent extraction of the virus from the first $(NH_4)_2SO_4$ precipitate. If the leaves that are being used in a virus preparation happen to have a low Ca content, there may be greater advantages in getting rid of the normal protein than in getting rid of the Ca, and this variation may make the preparation go more smoothly.

The C, H and N content of a virus preparation gives but little information about its purity; the P content, on the other hand, is a useful guide. In our earlier paper we gave 1·3–1·5% as the range within which the P contents of our preparations fell. In the more extended series of analyses that has since been carried out, on preparations dried at room temperature *in vacuo* over P_2O_5, we find that preparations containing less than 1·4% can always

be further fractionated. Under the conditions used by us the P method of Kuttner & Lichtenstein [1932] has a probable error of 5 %. With this uncertainty we look upon 1·5 % as the most probable value for the P content. An impure preparation of bushy stunt virus is likely to have a low P content, but, as we have already pointed out [Bawden & Pirie, 1938], its carbohydrate content will probably be high. The precipitate that separates from most preparations on prolonged dialysis at about *p*H 4·5 invariably has a high carbohydrate content, and it is rare to find a carefully purified preparation that contains as much as 7 % of carbohydrate estimated by the orcin method [Pirie, 1936] using glucose as a standard. The most usual value is 6 %. It is not possible to assess the significance of the difference between this figure and the range 11·4 to 7·1 % given for the carbohydrate content of centrifugally isolated virus by Stanley [1940], for he does not mention the method of estimation used, and the different methods of carbohydrate estimation give results that are not necessarily comparable.

The only difference we have found between preparations made by the method described in this paper and by that described earlier is that the former are more infective. In appearance, crystallizability, serological activity, analytical composition and sedimentation constant, they are indistinguishable. Recent measurements on preparations made by both methods give an $S^{20^\circ}_{W} = 130 \times 10^{-13}$. The discrepancy between this figure and that given by McFarlane & Kekwick [1938] is discussed in the Addendum to Bawden & Pirie [1943].

Table 1. *Comparison of activity of bushy stunt virus prepared by two methods*

Test	Method	Serological titre	Infectivity Av. no. of lesions per leaf at 10^{-4}	Infectivity Av. no. of lesions per leaf at 10^{-5}
1	Heating	1/600,000	35	9
	No heating	1/600,000	105	45
2	Heating	1/600,000	26	4
	No heating	1/600,000	96	40

In Table 1 purified preparations made by the two methods are compared, and it will be seen that virus made by the method described in this paper is at least 10 times as infective as virus made by the method involving heating. It is more difficult to be certain that the method causes no loss of infectivity. Comparisons between the infectivity of sap and the purified virus suggest that there is no loss, but it is well known that the constituents of sap can affect the number of lesions produced. The extent of this depends at least in part on the ratio of virus to normal plant constituents. This is illustrated in Table 2; during one purification the removal of contaminants has given an apparent increase in infectivity whereas in a second, with much more infective sap, it has not. To get the most highly infective preparations, sap should be worked up immediately, for, as we have already shown [Bawden & Pirie, 1940], during ageing *in vitro* the virus loses infectivity in much the same way as when heated.

Table 2. *Comparison of activity of sap and purified bushy stunt virus preparations*

Preparation	Serum precipitation end-point	Av. no. of lesions per leaf at 1 : 20	Av. no. of lesions per leaf at 1 : 200
Preparation 1:			
Sap from stalk and leaves	1 : 12	44	7
Virus preparation diluted to original vol. of sap	1 : 12	67	20

		Av. no. of lesions per leaf at 1 : 10	1 : 100	1 : 1000
Preparation 2:				
Sap from stalk and leaves	1 : 160	186	86	8
Virus preparation diluted to original vol. of sap	1 : 120	188	86	6

The virus content of the leaves from infected tomato plants is at least five times as great as that of the stems; the inclusion of stems in the preparation therefore has only a small effect on the yield and increases greatly the labour of mincing. The mincing is easier if the leaves have been frozen beforehand, but this freezing has no other advantage. We have, however, no evidence that it causes any loss of virus, for the salts and other constituents of sap protect the virus from the inactivation that takes place when virus solutions in water are frozen [Bawden & Pirie, 1938]. This phenomenon will be dealt with more fully in the following paper.

We have generally used tomato plants as a source of the virus and the yields have varied from 3 to 100 mg./l. of sap. The highest yields are obtained from young seedlings infected in the winter and the lowest from older plants infected in the summer. Purification is also easier from plants growing during the winter, as the normal plant constituents are then less troublesome to remove. We have also made preparations from *Datura stramonium* and *Nicotiana glutinosa*. *D. stramonium* is recommended by Stanley [1940] as giving a higher yield than tomato, but in our experience the sap of this plant is more troublesome to handle. The yield from *N. glutinosa*, as would be expected from a plant giving local lesions only, is very low. These yields all refer to the sap that is expressed after mincing in a domestic meat mincer. By more thorough grinding of the

leaf residue a further equal quantity of virus can be isolated. This phenomenon will be described in a later paper.

Tobacco mosaic virus

Precipitation with alcohol was one of the first steps in the method we previously described for the purification of tobacco mosaic virus. This successfully removed most of the normal leaf protein but was laborious.

In the preparations carried out during the past few years we have relied on precipitation at *p*H 3·3 to render the normal protein irreversibly insoluble. We have already stressed the aggregation that appears to accompany purification of the virus by our methods [Bawden & Pirie, 1937]; Bernal & Fankuchen [1941] suggest that this is a necessary corollary of purification and that the tendency towards linear aggregation of the particles is increased by the removal of some of the normal components of sap. Our view that preparations of tobacco mosaic virus are not homogeneous and consist of rods of equal cross-section but variable length, has received valuable support from Frampton's measurements [1942] of the lengths of virus particles measured by means of the electron microscope.

Preparation. Sap from infected plants is prepared by mincing and extraction with phosphate solution as in the method used for preparing bushy stunt virus. To each litre of fluid 250 g. of $(NH_4)_2SO_4$ is added and, after about ½ hr., the precipitate is centrifuged off; it is extracted with water until the extracts no longer have an obvious shimmer when stirred. In general four or five extractions are necessary, and the final volume of the extract is about one-quarter of the original volume of the sap. 1% NaOH is added in a fine stream with vigorous stirring to bring the *p*H to about 6·5, and the precipitation with $(NH_4)_2SO_4$ is repeated; if the supernatant is still dark brown a third precipitation is carried out. The precipitate is suspended in 10 times its bulk of water and centrifuged for 1 hr. at 3500 r.p.m., the dark brown precipitate is re-extracted with a smaller volume of water, and the virus precipitated by bringing the *p*H of the combined extracts to 3·3 by the addition of dilute HCl. After ½ hr. this precipitate is centrifuged off, washed once by mixing thoroughly with water and centrifuging again, transferred with the minimum amount of water to a dialysis sac, and dialysed against running tap water. After 24–36 hr. the *p*H will have risen sufficiently for the virus to dissolve, but at this *p*H, and in the presence of only traces of salt, most of the normal protein remains insoluble. The contents of the dialysis sac are diluted if necessary and centrifuged; centrifugation is likely to be very slow if the fluid contains more than 1% of solid. If the precipitate is bulky it should be re-extracted. This method of dissolving the acid precipitate of virus has been adopted because it avoids the risk of inactivating the virus in the small regions of high *p*H which are almost unavoidable if alkali is added directly. This dialysis also brings about a useful separation of the virus from some normal constituents of the sap.

The further treatment depends largely on the appearance of the fluid. If young tomato or white burley tobacco plants have been used it will be nearly colourless, but with older plants it will be brown. If the fluid is brown the *p*H should be adjusted to 6·5 and the $(NH_4)_2SO_4$ precipitation repeated. Pale or colourless preparations are reprecipitated with acid and dialysed thoroughly against distilled water; the dialysis sac should be shaken gently. If necessary the fluid is centrifuged and is then left undisturbed in the ice chest for a few days for the separation of the lower, liquid-crystalline, layer. With aucuba mosaic this should separate from a solution containing 2% of virus, but with the other strains 3% is generally necessary. As in preparations made by the previous method the lower layer is, from a chemical standpoint, the purer and has a higher serum precipitation end-point than the upper, but it is slightly less infective. This method of preparation gives good results with either tobacco or tomato plants.

The recovery of virus by this procedure is satisfactory and there is no evidence of any great inactivation. Thus from 1600 ml. of tomato sap 610 mg. of aucuba mosaic virus was isolated in the form of 'Bottom layer' and 435 mg. as 'Top layer', that is to say 0·66 g. was isolated for each litre of sap. The serum precipitation end-point of the sap was 1:1500. If the 0·66 g. represented complete recovery of virus this corresponds to an end-point of $2{\cdot}3 \times 10^6$, and any loss during the preparation necessitates the assumption of an even lower end-point. The 'Top' and 'Bottom' layers, on the other hand, had serum precipitation end-points of $1:4 \times 10^6$ and $1:6 \times 10^6$ respectively. For infectivity measurements it was assumed that the sap contained 0·66 g./l. and dilutions were made accordingly. In Table 3 the number

Table 3. *Average number of lesions per leaf for sap and purified aucuba mosaic virus*

	Dilution of virus		
	$1:10^5$	$1:10^6$	$1:10^7$
Sap	76	21	1
'Bottom layer'	49	15	0
'Top layer'	52	12	2

of lesions found is set out. These results are most simply explained by postulating that linear aggregation of the virus particles during purification has increased the serum precipitation titre, whereas the fall in infectivity, which would be expected to accompany such aggregation, has been masked by an apparent increase in infectivity due to the removal of normal sap constituents.

The serum precipitation end-points given above for aucuba mosaic virus are similar to the end-points 6×10^6 to 10^7, found for this strain of virus in 1937. With tobacco mosaic virus, on the other hand, we are unable to duplicate the old high values for the end-point. The strains that we handle now give precipitation end-points of about 10^6 instead of the old values of 6×10^6 and 10^7. This change is probably due to the inadvertent selection of a new strain of tobacco mosaic virus, but we have no definite evidence of this.

SUMMARY

Methods, requiring only low-speed centrifuges, are described for the purification of tomato bushy stunt and tobacco mosaic viruses.

These preparations appear to contain virus that is weight for weight as infective as that in clarified sap. There is evidence, however, that the tobacco mosaic virus particles have undergone some aggregation.

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The Inactivation of Tomato Bushy Stunt Virus by Heating and Freezing

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We have often stressed the fact that there is some uncertainty over the exact name most suitable for use when speaking of purified virus preparations. It can hardly be questioned that the specific nucleoproteins, isolated from infected plants, bear some relationship to the viruses themselves, and we have used the term viruses when referring to these proteins. The exact relationship between these proteins and the viruses as they are produced in the infected cells, however, is by no means clear.

All the viruses with which we have worked can be rendered non-infective without changing their serological reactions, and such inactive virus preparations have physical properties at present indistinguishable from those of active virus. Because of this, physical and serological tests cannot be taken as proving the homogeneity of virus preparations. With viruses such as potato '*X*' and tobacco mosaic complete loss of infectivity without loss of serological activity has been produced by X-rays, ultraviolet light, H.CHO, H_2O_2 and HNO_2, but the activity and homogeneity of purified preparations of these viruses is affected by the tendency of the particles to aggregate linearly [Bawden & Pirie, 1937; 1938*b*]. Tomato bushy stunt and tobacco necrosis viruses do not appear to aggregate in this way, but they are more easily rendered non-infective by ageing *in vitro*, moderate heating and treatment with alkali.

In the method we described for the isolation of a crystalline protein from plants suffering from bushy stunt, the sap was heated to about 60° to facilitate clarification [Bawden & Pirie, 1938*a*]. We now know that this treatment must have led to considerable inactivation, and Stanley [1940] showed that more infective preparations could be produced by differential centrifugation. More infective preparations can also be made by precipitation methods if heating is omitted. The method of isolation we describe [Bawden & Pirie, 1943] gives a crystalline product that weight for weight is as infective as the virus in clarified infective sap. At the moment this is the only test available for estimating full activity, but it is not necessarily a valid one. First, it is possible that inactivation without loss of serological activity occurs in the living plant, so that the virus in the sap is already a mixture of infective and non-infective particles. Secondly, sap may contain some inhibitor of infectivity; if this is so, some inactivation could occur during purification but pass unnoticed, as it would be balanced by the removal of the inhibitor. Until more sensitive methods for detecting activity are developed, so that it can be shown that one virus particle can cause infection, it is not likely to be possible to prove that any virus preparation is homogeneous.

The main conclusions of our earlier paper on tomato bushy stunt virus were confirmed by Stanley [1940]. There are some apparent disagreements, however, and the significance of these is discussed in this paper, which deals mainly with the effects of heating and freezing the virus. The methods of testing were similar to those described previously [Bawden & Pirie, 1938*a*].

EXPERIMENTAL

The effect of heating purified virus preparations

The actual thermal inactivation point of bushy stunt virus, i.e. the temperature at which 10 min. heating gives complete inactivation, has varied in different tests between 70 and 85°. The variation may depend partly on the concentration of the virus solution heated, but it appears chiefly to be determined by the susceptibility of the host plants used. The same inoculum may produce 10 or more times as many local lesions in one lot of test *Nicotiana glutinosa* plants as in another, and it is obvious that the term complete inactivation has no real meaning in these conditions, for whether or not infection is obtained depends first on the concentration of the inoculum and secondly on the susceptibility of the host. In general, the younger the *N. glutinosa* plants the more lesions per leaf are obtained. In moderately developed plants, there is an increase in the susceptibility of leaves from the base to the top so that the same inoculum may only give one or two lesions on a basal leaf but give 50 or 60 on leaves nearer the top. This is in striking contrast to results with tobacco mosaic virus on the same host where there is a gradient of increasing susceptibility from the top to the base. With tobacco mosaic virus the differences are less pronounced, but if solutions of the two viruses are rubbed on opposite halves of the same leaves, those at the top of the plant will often give more lesions with bushy stunt than with tobacco mosaic virus, whereas those at the base will bear great numbers if rubbed with tobacco mosaic but few or none with bushy stunt virus.

Table 1. *Effect of heat on purified bushy stunt virus made with and without previously heating infective sap*

	Virus from unheated sap		Virus from heated sap	
Temp.	Serological titre	Av. no. of lesions per leaf at 10^{-4}	Serological titre	Av. no. of lesions per leaf at 10^{-3}
Unheated	1/600,000	83	1/600,000	78
45°	1/600,000	58	1/600,000	55
50°	1/600,000	33	1/600,000	36
55°	1/600,000	16	1/600,000	19
60°	1/600,000	5	1/600,000	12
70°	1/600,000	3	1/600,000	8
80°	1/600,000	1	1/600,000	4
85°	*Nothing at 1/5000	0	*Nothing at 1/5000	0

* Tested after centrifugation; the infectivity results are expressed as the average number of lesions per leaf on *N. glutinosa*.

Influence of temperature. Table 1 shows the effect of 10 min. heating at various temperatures on two preparations of virus made in different ways, one in which the infective sap was previously heated and the other not. Both were heated as 0·1 % solutions in 0·1 *M* phosphate buffer at *p*H 6, the preparation from heated sap was tested for infectivity without further dilution and the other was diluted 1:10 so that the two were comparable in infectivity. Although loss of infectivity is only complete after heating at 85°, some infectivity is lost at temperatures as low as 50° and most of it below 60°. By contrast, the precipitation titre is unaffected by heating up to 80°, but a little heating above this is sufficient to destroy the serological reactions. This destruction is accompanied by the separation of a coagulum. Thus there is a wide range in the temperatures over which loss of infectivity can be measured, but loss of serological activity resembles other examples of protein denaturation in proceeding at a measurable rate only within a narrow temperature range. In this respect tomato bushy stunt virus resembles the tobacco necrosis viruses and differs from other viruses investigated, which lose both infectivity and serological activity *pari passu* in a narrow temperature range [Price, 1938; 1940; Bawden, 1941]. Virus preparations that have been largely inactivated by heating during the preparation lose their residual activity according to much the same law that governs the loss of activity by fully active preparations.

Influence of duration of heating. Preparations with no demonstrable infectivity can be produced by heating at temperatures well below the thermal inactivation point, but prolonged heating is necessary. Thus in *p*H 6 phosphate buffer at 50°, a 0·5 % virus solution lost half of its infectivity in 15 min. three-quarters in 1 hr. and all in 24 hr.; at 60° the solution was non-infective after 1 hr. and at 70° after 30 min. Such heated preparations do not change their appearance, they retain their full serological activity and crystallize as easily and in the same form as unheated ones.

Table 2. *The infectivity of fractions from a mixture of infective and non-infective sap*

	Av. no. of lesions per leaf at	
Virus preparation	10^{-4}	10^{-5}
1. Active virus	80	41
2. Inactive virus	0	0
3. Mixture of 1 part of no. 1 with 4 parts of no. 2	46	18
4. 1st crystalline fraction from no. 3	43	15
5. 2nd crystalline fraction from no. 3	38	16

We have attempted to fractionate preparations of partially inactivated virus by crystallization but have had no success. As many as five crystalline fractions have been made from preparations derived

from heated sap, but they all had similar infectivities. Artificial mixtures of infective and non-infective virus have also been made and subjected to fractional crystallization, without any separation. The results of one such test are given in Table 2.

Influence of pH. The virus is most stable around *p*H 6, but heating at any *p*H value between 4 and 9 can cause loss of infectivity without a corresponding loss of serological activity. At *p*H 4, however, denaturation occurs with much less heating than at higher values. In *p*H 6 *M*/10 phosphate buffer, 0·1 % solutions become opalescent after 10 min. at 80° and still further heating is necessary to cause precipitation. In *p*H 4 *M*/10 phthalate buffer, on the other hand, 0·1 % solutions are opalescent after 10 min. at 60° and 10 min. at 70° causes precipitation.

Heating for 10 min. at 60° and *p*H 4 has no effect on the serological activity, but material that is flocculated specifically by virus antiserum can be removed from such solutions by centrifugation for a few minutes at 5000 r.p.m. Heating for longer periods in these conditions increases the amount of this material, but it also causes the spontaneous separation of a precipitate. At *p*H 6 more intense heating is needed to produce this effect. Table 3

Table 3. *Effect of heating purified bushy stunt virus solutions at* p*H 6 and 80° for various times on the serological activity*

Time of heating min.	Appearance of heated fluid	Serological titre: Before centrifuging	Serological titre: After centrifuging
0	Clear	1/320,000	1/320,000
7·5	Slightly opalescent	1/320,000	1/160,000
15	Opalescent	1/160,000	1/ 40,000
30	Opalescent and slight flocculation	*1/ 80,000	1/ 5,000
60	Considerable flocculation	*1/ 10,000	No ppt. at 1/2,000

* Tests made on supernatant fluid after floccules had settled.

shows the results of heating 0·1 % solutions of virus in 0·1*M* phosphate buffer at *p*H 6 for various times at 80°.

The effect of miscellaneous treatments. The nature of the changes in the virus particle when it is rendered non-infective by an amount of heating insufficient to cause denaturation is unknown. As treatment with H_2O_2 has a similar effect, it was thought that oxidation might be responsible. Tests were made therefore to compare the effects of heating virus solutions at *p*H 6 in air and *in vacuo*, but no differences were found. In one such test, the unheated control gave an average number of lesions per leaf at 10^{-3}, 10^{-4} and 10^{-5} of 79, 57 and 22, while those heated in air and *in vacuo* for 10 min. at 60° gave 19, 6 and 1·5 and 21, 8 and 2 respectively. Similarly, attempts to preserve the infectivity of bushy-stunt virus in expressed sap by the addition of reducing agents has failed. Indeed, the addition of 5 % Na_2SO_3 caused a rapid inactivation of a type resembling that caused by heating or ageing. Within 24 hr. at room temperature the sap had lost its infectivity, but from it we isolated a crystalline and serologically active product indistinguishable from a normal virus preparation except for its complete lack of infectivity. This type of inactivation is not reflected by any change in the sedimentation constant or in the phosphorus or carbohydrate contents. This is shown in Table 4, where sedimentation constants and analytical figures for virus preparations

Table 4. *Sedimentation constants and carbohydrate and phosphorus contents of active and non-infective purified virus preparations*

		Phosphorus %	Carbohydrate %
Fully active virus	$S_{20} = 130{\cdot}9 \times 10^{-13}$	1·47	6·0
Virus inactivated by heating at 59–60°	$S_{20} = 130{\cdot}1 \times 10^{-13}$	1·44	6·5
Virus inactivated by ageing	$S_{20} = 130{\cdot}2 \times 10^{-13}$	1·53	6·6

made non-infective by various treatments are given. The analytical methods were those used in our earlier paper. Sedimentation constants were measured by Dr A. G. Ogston and are set out in the Addendum to this paper.

The effect of freezing purified virus preparations

We have already briefly noted the inactivation of purified preparations of bushy stunt virus by freezing [Bawden & Pirie, 1938*a*]. These experiments were made only with dialysed solutions containing 5–20 g./l. and near the isoelectric point [*p*H 4·11, McFarlane & Kekwick, 1938]. Stanley [1940] found that freezing infected leaves did not destroy the virus, and we have also found that freezing plant tissues or expressed infective sap does not inactivate. We have now made experiments on the effects of freezing the virus in different conditions and find that the rate and extent of inactivation are affected by a wide range of variables. The variables we have considered are concentration of virus, *p*H, duration of freezing, temperature to which the preparation is taken, and the presence of other materials.

Unless otherwise stated, the virus preparations used in this work were purified and dialysed. So far as we know they were free from any material

other than virus. Some preparations were made by the old method, which involved heating the sap to 60°, and some by the method described in the preceding paper. Several comparisons have been made in which these two products were frozen under the same conditions and no differences have been found in their behaviour.

No experiments made at temperatures below −20° are reported in this paper. Thus, freezing means the more or less complete separation of crystalline ice, and our experiments have little in common with those [e.g. Luyet, 1937; Luyet & Thoennes, 1938] in which vitrification is induced by sudden, extreme cooling.

All the experiments on freezing were carried out in 3 × ⅜ in. test tubes. The mixture of virus solution and the other components of the system was carefully pipetted in to form a pool at the bottom of the tube without wetting the walls; in general 0·1–0·4 ml. was frozen. A metal stand, previously cooled, held the tubes in a nearly horizontal position in a part of the refrigerator at the required temperature. Freezing generally occurred after a few minutes, but if it did not the mixture was seeded with about 0·005 ml. of ice carried on a cooled rod. In some experiments, especially those in which only short periods of freezing were used, the tubes were partly immersed in an ice and alcohol freezing mixture at the required temperature. This was in a glass vessel so that the moment of freezing could be easily noted. No differences have been detected between mixtures freezing at the slightly different rates that these different procedures probably involve.

After thawing, the amount of insoluble material was estimated by eye; apparently complete precipitation was called 3, substantial precipitation but a still opalescent supernatant 2, and a trace only of precipitate was called 1. The *p*H of the mixture was brought to 5·5 by the addition of *p*H 6·0 phthalate buffer or, in the experiments run at low *p*H's, phthalate to which an excess of NaOH had been added. Before testing for serological activity and infectivity the mixture was diluted with 0·9% NaCl solution so that its virus content, in terms of the initial amount of virus present, was 0·2 g./l., and centrifuged.

Influence of the concentration of virus. Table 5 shows that, at *p*H 4·1 and 2 hr. freezing, inactivation is complete only when the virus concentration exceeds 10 g./l. It also shows that the inactivated virus becomes insoluble. The results here are in good agreement with those of Nord and his collaborators in a series of papers on 'cryolysis', e.g. Nord [1933], Nord & Lang [1935], Holzapfel & Nord [1938 *a*, *b*] and Kausche & Holzapfel [1940]. They find, using a wide range of colloidal materials, that changes leading to an aggregation of the particles are favoured by an increase in the concentration of the solution that is being frozen, and that dilute solutions tend to disaggregate. An aggregation leading, as with bushy stunt virus, to the separation of a precipitate was not observed in Nord's systems, but it is a very well-known result of freezing other systems; soil [Jung, 1931] and normal leaf protein [Bawden & Pirie, 1938*c*] may be mentioned as examples, and the similar behaviour of agar is the basis of the methods used for its commercial preparation.

Table 5. *Effect of freezing purified preparations of virus at* p*H 4·1; influence of virus concentration*

Conc. of virus (g./l.)	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point
20·0	3	No ppt. at 1 : 1,000
10·0	3	1 : 1,000
5·0	3	1 : 4,000
2·5	2	1 : 32,000
1·25	1	1 : 128,000
Unfrozen control	0	1 : 320,000

The virus was dissolved in *M*/20 phthalate buffer solution at *p*H 4·1 and the samples were held frozen at −10° for 2 hr.

Influence of p*H.* Although Table 5 shows that concentration is an important factor in determining the degree of inactivation on freezing, *p*H is even more important. When solutions of the same concentration as those listed in Table 5 are frozen at *p*H 3·5, for example, even the most dilute is completely inactivated. Inactivation also occurs in solutions more dilute than these. At *p*H 5, on the other hand, inactivation is incomplete even after 12 hr. freezing. The results of an experiment in which a series of tubes containing 1 g. virus/l. were frozen at different *p*H values are set out in Table 6. It is clear from this experiment that an acid solution favours inactivation.

Table 6. *Effect of* p*H on purified virus preparations*

*p*H	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point
3·5	3	Less than 1 : 5,000
4·0	3	1 : 40,000
4·5	2	1 : 80,000
5·0	1	1 : 200,000
5·5	0	1 : 400,000
6·0	0	1 : 400,000
Unfrozen control	0	1 : 400,000

1 g./l. solutions were held frozen at −10° for 12 hr. in *M*/20 phthalate buffer.

Influence of duration of freezing. The time for which the material is held frozen can also affect the extent of inactivation. In general, the amount of inactivation increases with the duration of freezing. Clear-cut results can be obtained when the differences in duration of freezing are large, but, as perhaps might be expected, we have been unsuccessful in getting consistent results when comparing

times of freezing that do not differ greatly from one another. Table 7 illustrates these phenomena at *p*H 4·5; at other *p*H values the extent of inactivation also depends on the duration of freezing. Under the conditions of these experiments all the liquid has apparently solidified within a few seconds of the first signs of ice formation, for there is considerable supercooling and the volumes of fluid are small, whereas the surface exposed to cold and the heat capacities of the vessel and stand are large. This apparently almost instantaneous solidification, however, does not necessarily imply immediate complete freezing. Moran [1926], by following the volume changes in a gel containing 44 % of gelatine, showed that freezing was only complete after 26 days at −11°, and Kistler [1936] reached a similar conclusion with emulsions of water and toluene. The latter reviews the literature of the freezing of colloids as does Blanchard [1940]. Our mixtures have a much lower colloid content than those used in the experiments of the workers referred to above, and we have no evidence that incomplete inactivation in short exposures to cold is in fact due to incomplete freezing at −10°. However, this is the simplest explanation of our results and may well be adopted until some evidence against it is forthcoming.

Table 7. *Effect of duration of freezing on purified virus preparations*

Duration of freezing (min.)	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point	Av. no. of lesions per leaf at 10^{-4}	Av. no. of lesions per leaf at 10^{-5}
0	0	1:320,000	133	57
10	0	1:320,000	131	31
40	0	1:160,000	124	18
225	1	1:160,000	113	28
450	1	1:160,000	47	15
1465	3	1: 40,000	5	1
1930	2	1: 80,000	5	1
2410	3	1: 20,000	3	1

0·1 ml. lots of 10 g./l. virus solution in *M*/30 phthalate buffer at *p*H 4·5 were frozen for the time stated.

Influence of freezing to different temperatures and in the presence of various other materials. There are three obvious ways in which another solute may affect the inactivation of bushy stunt virus by freezing. First, if the eutectic temperature of the substance added is lower than the temperature to which the mass is frozen some of the solution will remain unfrozen; if enough of this solution is available to dissolve the virus, that is to say if the ratio of solute concentration to virus concentration is high enough, there is no reason why the virus should be affected by the freezing. In the second place, it may delay the establishment of complete freezing throughout the mass of the material; if a substance is acting in this way an increase in the duration of freezing should neutralize the effect. Finally, the solute may combine with the virus or precipitate it from solution; the complex or the precipitate may have a different stability from the normally dissolved virus when the surrounding water is frozen. Phthalate buffer was present in all the freezing experiments described in this section, and also in the three described above, in which it was necessary to control the *p*H. The solubility of phthalate is so low that we have no reason to think that it exerts any protective action at −10° nor have any experiments suggested it. All the data on eutectic temperatures and the concentration of the eutectic are taken from Landolt and Bornstein's Tables.

Table 8 illustrates the first mechanism. Probably because of the length of time for which the samples were held frozen these results were clear cut, and the virus was either inactivated or else retained its infectivity and serological activity substantially unimpaired. Table 9 illustrates the first and second mechanisms, KNO_3 delays the inactivation somewhat, and NaCl, in spite of its low eutectic, does not, at this concentration, protect indefinitely. The amount of water that would be held unfrozen at −10° by the 0·27 mg. of NaCl that is in these 0·1 ml. test samples is 0·81 mg. This amount is apparently insufficient for the permanent stability of 1 mg. of virus under these conditions. In another experiment with 10 g. virus/l. at *p*H 4·5, inactivation was not appreciable in 24 hr. at −10° in the presence of 0·56 % NaCl but was nearly complete with 0·17 %. The results in a series of tubes containing the latter mixture were very variable at shorter times of freezing; this suggests that we are here at about the limit at which the amount of water held unfrozen is insufficient to preserve the virus. This hypothesis offers an explanation of the greater stability of dilute virus solutions for, other things being equal and in the presence of unavoidable traces of salt, more water is available in them for each mg. of virus. The protective action of glucose is also shown in Table 9; with as little as 0·1 % glucose there is some delay in the inactivation.

Many attempts have been made to test the protective effect of salts which have eutectic temperatures above the temperature of freezing, and which will precipitate the virus. Crystals with the usual dodecahedral form can be made by precipitating the virus at 0° with $FeSO_4$, $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $Na_2S_2O_3$; the eutectic temperatures of these salts are −1·8, −3·9, −6·5, −10·5 and −11·0° respectively. In some experiments these salts have been found to exert a greater protective effect than salts such as $CuSO_4$, KNO_3, KCl and $BaCl_2$ that do not precipitate the virus at 0°, but we have not been able so to define the conditions that consistent results can be obtained.

Table 8. *Effect of salts with different eutectic temperatures on purified virus preparations*

Salt	Eutectic temp.	Conc. at the eutectic of salt/100 g. of water (g.)	g. of salt/100 g. of water in the solution frozen	Amount of material insoluble after freezing at (arbitrary units, see p. 73)		Activity after thawing from	
				−10°	−20°	−10°	−20°
KNO_3	− 2·9°	12·2	4·9	3	3	−	−
KCl	−11·1°	24·0	9·6	1	.	+	.
NH_4Cl	−15·8°	22·9	9·2	0	3	+	−
NaCl	−21·2°	28·9	11·5	0	.	+	.
$MgCl_2$	−33·6°	27·5	11·0	0	0	+	+

Each test was carried out on a solution containing 40% of the eutectic concentration of the salt, 1·4 g. virus/l. and *M*/10 phthalate buffer at *p*H 4·4. In all cases the duration of freezing was 68 hr.

Table 9. *The protective effect of different materials on purified virus preparations*

In all these experiments 0·1 ml. lots of a 10 g./l. solution of virus in *M*/25 phthalate buffer at *p*H 4·0 were kept frozen at −10°. In addition the fluids contained the following substances.

Time of freezing	Salt added	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point	Av. no. of lesions per leaf at 10^{-5}	Av. no. of lesions per leaf at 10^{-6}
1 min.	No addition	1	1:320,000	63	8
	1·6% KNO_3	0	1:320,000	29	7
	0·27% NaCl	0	1:320,000	30	12
	1·5% glucose	0	1:320,000	49	8
1 hr.	No addition	3	No ppt.	7	1
	1·6% KNO_3	2	1: 40,000	20	1
	0·27% NaCl	0	1:320,000	20	5
	1·5% glucose	0	1:320,000	23	3
24 hr.	No addition	3	No ppt.	0	0
	1·6% KNO_3	3	No ppt.	0	0
	0·27% NaCl	1	1: 80,000	3	0
	1·5% glucose	0	1:320,000	18	5
0	No addition	0	1:320,000	48	22

Effect of freezing infective sap

In infected leaves and in expressed sap, bushy stunt virus is apparently unaffected by freezing for days or even weeks. The results that have been described above give ample reason for this. Even the most highly infective sap has only a quarter of the virus concentration that is necessary for inactivation at the isoelectric point. Tomato sap has never been found with a *p*H lower than 5·6, and the salts and sugars in the sap supplement these other protective effects. After dialysis, infective sap is still not inactivated if it is frozen at its own *p*H, but it is inactivated if acidified before freezing; this is shown in Table 10. The sap from minced tomato leaves was frozen, centrifuged and dialysed for 48 hr. against frequently changed distilled water. After centrifuging again, it contained 1·8 g. solids/l. and was at *p*H 6. Samples were adjusted to *p*H 3, 4 and 5 with *N*/10 H_2SO_4, and 2 ml. lots were frozen at −10° for 14 hr. They were all centrifuged after thawing, although there was no precipitate in the one at *p*H 5, and tested. It is clear that with this dilute virus solution, inactivation took place only at *p*H 3; the serum precipitation end-point suggests that this dialysed sap contained about 0·02 g. virus/l.

Table 10. *The effect of* p*H on the freezing of infective sap*

*p*H of sap	Serum precipitation end-point		Av. no. of lesions per leaf	
	Frozen	Unfrozen	Frozen	Unfrozen
5	1:8	1:8	64	85
4	1:8	1:8	68	91
3	No ppt.	1:8	6	65

Virus is also protected by the colloidal constituents of sap from inactivation by freezing. The effect is not very definite, but a solution containing 5 g. virus/l. and at *p*H 4·0 was only half inactivated in 18 hr. at −9°, when half of the final volume of solution consisted of dialysed healthy sap prepared as in the preceding experiment. When the proportion of sap colloids was only one-sixth the activity fell to one-eighth, and with less sap than this there was no protection. The colloidal material present in sap prepared in this way is largely polysaccharide,

but, since it also contains 3–4 % of N, a few experiments on protection by proteins have been made. Dialysed preparations of rabbit-serum albumin and pseudoglobulin were used and, as Table 11 shows, they had a substantial protective effect.

Table 11. *The protective effect of proteins on virus exposed to freezing*

Each mixture contained 2 g. of virus/l. dissolved in *M*/20 *p*H 3·5 phthalate buffer solution, the addition specified was also made. All except the control were kept frozen at −10° for 14 hr.

Addition	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serum precipitation end-point	Av. no. of lesions per leaf	
			10^{-4}	10^{-5}
None	3	0	0	0
1·6 % albumin	2	1: 80,000	6	7
0·5 % albumin	3	1: 40,000	7	0
1·6 % pseudo-globulin	1	1:320,000	48	11
Unfrozen control	0	1:320,000	169	53

Loss of infectivity without loss of serological activity

In general, loss of infectivity in frozen samples of bushy stunt virus is accompanied by a corresponding loss of serological activity and by the production of insoluble materials. However, inactivation by freezing, as by heating, is a complex process, and loss of infectivity is produced by the earliest changes. It is more difficult to make serologically active but non-infective preparations by freezing than by heating, but the results in several of our tables show that some of the frozen samples have suffered a greater loss of infectivity than of serological activity.

As with so many other aspects of this study, conditions cannot be defined which will always give a separation of the two activities on freezing. It is probable that the lack of fine control over the rate of freezing accounts for much of the variability. The most consistent results have been obtained with 0·2 % virus solutions in *M*/10 phthalate or acetate buffer at *p*H 4·25 kept at −10° for 2 hr. In about half the tests under these conditions, preparations have been non-infective although fully active serologically, and no other conditions have been found that give a higher proportion of successes. Inactivation of this type has no effect on the sedimentation constant. When such non-infective preparations were set to crystallize with $(NH_4)_2SO_4$, most of the material crystallized in the form of the usual dodecahedra, but the supernatant fluids showed a slight shimmer when shaken. Such an effect has not been noticed during the crystallization of normal preparations of bushy stunt virus, although it has been during attempts to crystallize serologically active preparations made non-infective by heating in alkaline solution. On long standing in the presence of $(NH_4)_2SO_4$ a few birefringent crystals have separated from virus preparations made non-infective, but not denatured, by freezing.

The fact that freezing can produce this type of inactivation needs to be remembered when virus preparations are being made from frozen leaves or sap, for much of the non-infective virus is indistinguishable, by the criteria we have used, from fully active virus. It is perhaps unlikely that inactivation of this type would occur under the conditions obtained in frozen sap, but if it did, infective and non-infective virus would not be separated by the ordinary purification procedure.

DISCUSSION

So many treatments can cause loss of infectivity without producing any appreciable changes in physical, chemical or serological properties, that attempts to assess the homogeneity of purified virus preparations are likely to give indecisive results until much more sensitive methods of testing for infectivity are discovered. The local lesion method is adequate for comparing the relative activity of different preparations, but it tells little or nothing about the absolute activity. The fact that the same virus preparation can give widely varying numbers of lesions on different plants of the same species, or on different leaves of the same plant, shows that the number of lesions formed in any one test bears no fixed relationship to the quantity of active virus in the inoculum. Even when using the most sensitive leaves, however, some thousands of times the amount of virus has to be applied that would be needed in theory if one virus particle can cause infection. This discrepancy may arise because of the wastage of inoculum in the relatively crude methods of inoculation used, or it may be that large numbers of active virus particles are needed to cause one lesion. The latter possibility could be tested by the method used with vaccinia [Parker, 1938; Haldane, 1939], that is to say, by determining the proportion of inoculations that fail to give any lesions when different amounts of virus are present in the inoculum. An equally probable explanation, however, is that even the best preparations of virus yet made consist largely of virus that has lost infectivity but not serological activity. Although this type of inactivation is a complicating factor in work on the purification of viruses, its occurrence makes viruses particularly suitable material for studying the earliest changes that lead to denaturation. With proteins that have no specific biological activities, changes can only be detected when the physical, chemical or serological properties have been altered measurably.

Ideally, a pure virus preparation would consist of particles all identical in size, constitution and ability

to cause infection, but there is no evidence that this ideal has ever been achieved. Deviations from it can arise in four main ways. First, the purified preparations may still contain material that is either a constituent of the normal host or a product of infection that is inessential for infectivity. The detection of such contaminants should not present serious problems, and it is probable that preparations of some viruses have been made substantially free from them. In this connexion it should be remembered that, although it may be legitimate to regard the more active of two preparations with equal solid content as the more pure, all contaminants do not necessarily reduce infectivity and serological activity [Pirie, 1940]. Secondly, even when all the particles in a virus preparation are fully active, these particles may be of different kinds. Most preparations of tobacco mosaic virus fall into this category, for even cultures derived from single local lesions are often mixtures of virus strains [Jensen, 1936]. Thirdly, during the course of isolation some or all of the virus particles may have become modified so that, although the final product consists solely of virus particles or of particles derived from them, these may vary in activity. The detection of inactive contaminants arising in this manner presents serious problems only when the modification is slight. Finally, all the virus particles in an infected leaf may not be equally infective although inseparable by the available methods. Particles less active than the ideal may be stages in the production of fully active virus or they may be particles that have suffered inactivation. The first alternative would offer a ready explanation for Spencer's [1941] observation that, weight for weight, tobacco mosaic virus from recently infected plants is less infective than virus from plants infected for longer periods. The second might also be expected as it is analogous to the type of inactivation that occurs in sap extracted from plants suffering from bushy stunt or tobacco necrosis.

In the past the thermal inactivation point has been widely used as a property for characterizing individual viruses, and with most viruses different workers have usually agreed within narrow limits about the temperature at which 10 min. heating causes loss of infectivity in expressed sap. The data on bushy stunt and tobacco necrosis viruses, however, have been conflicting. Provided the *p*H and other conditions of heating are controlled, reproducible results are readily obtained with potatc virus '*X*' and tobacco mosaic virus. But with the same preparation of bushy stunt virus, the thermal inactivation point may vary over 15° in successive tests. These differences seem to arise because of the different coefficients of thermal inactivation for the different types of viruses. Loss of infectivity with virus '*X*' and tobacco mosaic virus is closely linked with denaturation and coagulation of the protein and it has a large temperature coefficient, so that the precise temperature to which the preparation is heated is of the greatest importance in determining loss of infectivity. With bushy stunt virus, on the other hand, loss of infectivity is not closely linked with denaturation and the former has a small temperature coefficient. Thus the exact temperature of heating is of little more importance in determining the apparent point of inactivation than other factors such as concentration of inoculum and susceptibility of the test plants. With such viruses, determinations of the thermal inactivation point are obviously of reduced value as a diagnostic character. Measurements of the rate of inactivation at different temperatures, however, may become of value in differentiating between those viruses with widely different coefficients of thermal inactivation. In our earlier paper on bushy stunt virus, we stressed inactivation by freezing as a property that sharply differentiated this from the other viruses we had studied, for none of the others had been affected by freezing. However, we did not then realize that the inactivation of bushy stunt virus was so dependent on the precise conditions of freezing, and other viruses may be similarly inactivated if the freezing is done under more critical conditions.

SUMMARY

Tomato bushy stunt virus loses its infectivity when heated insufficiently to cause denaturation and loss of serological activity. The temperature coefficient for loss of infectivity is small and for loss of serological activity is large. The amount of heating needed for denaturation varies with the *p*H.

No differences have been found between the chemical and physical properties of non-infective, but serologically active, material and those of fully active preparations.

The rate of inactivation by freezing is increased by increases in the concentration of the virus, in the duration of freezing and in the acidity of the fluid. The virus is protected from inactivation by salts and some other substances. The efficiency of different salts depends on the salt:ice:water eutectic temperature.

In general, loss of infectivity is accompanied by the separation of a precipitate and loss of serological activity, but in some conditions freezing destroys infectivity without altering serological activity.

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Note added 19 Nov. 1942. Since this paper was written a comprehensive review (Breedis, C. [1942], *J. exp. Med.* **76**, 221) on the effects of freezing on a variety of biological materials has appeared. It also contains experiments showing the importance of controlling the rate of freezing in the destruction of leukaemia cells.

Addendum: Examination of Bushy Stunt Virus in the Ultracentrifuge

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with the technical assistance of W. Weinstein

Nine samples of bushy stunt virus were examined in a Svedberg oil-turbine ultracentrifuge by the 'diagonal schlieren' method [Philpot, 1938]. The speed of rotation was 300–310 rev./sec.; the solutions, of concentration 0·35 or 0·25 % in 0·1 *M* NaCl with 0·01 *M* buffer (acetate *p*H 4·7 or phosphate *p*H 6·8), were contained in a 12 mm. cell.

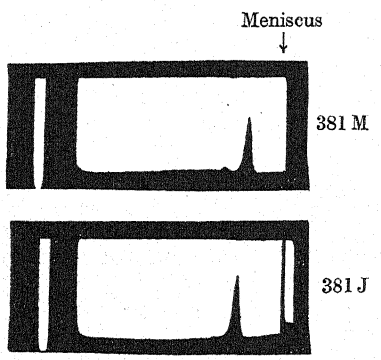

Fig. 1.

The results and details are given in Table 1 and specimen schlieren diagrams in Fig. 1.

All the preparations (except 381 M which contains a small amount of a more rapidly sedimenting component) appeared to be homogeneous. In three cases the fraction of the total refracting protein which appears in the boundary was measured by the method of Philpot [1939]; these figures are given in Table 1; but because of the sharpness of the boundaries, and the smallness of the areas measured, the figures are not very accurate; they indicate that the samples are largely homogeneous.

The sedimentation constants obtained here agree with the mean value obtained in five other laboratories on Lauffer & Stanley's [1940] virus, including the value of 133 obtained by McFarlane. It differs significantly from the value of 146 which McFarlane & Kekwick [1938] obtained on an earlier virus preparation by Bawden & Pirie [1943], but I am asked by these authors to state that this value is probably too high because of irregularities of temperature in the rotor of their Svedberg equilibrium centrifuge, which they had not suspected at the time. Recently the same virus preparation (bushy stunt 515 J of Bawden & Pirie) has been examined in both laboratories (0·25 % in acetate buffer *p*H 4·0 of ionic strength 0·02) and the mean of three values (Ogston) was 129, while the mean of four values (McFarlane & Kekwick) was 133.

Table 1. *Examination of bushy stunt virus in the ultracentrifuge*

Run	Index no.	Type of product	Conc. %	pH	$S^{20°}_{W} \times 10^{-13}$	Fraction in boundary
270	381J	Unheated	0·25	4·7	135·0	0·87
269	368G	Unheated	0·25	4·7	130·3	—
274	381K	Unheated	0·25	4·7	133·8	—
275	431B	Heated after purification	0·25	4·7	130·1	—
273	381M	Heated after purification	0·25	4·7	133·4	—
					225	*ca.* 0·05
266	363G	Prep. from heated sap	0·25	4·7	130·8	0·93
267	363G	Prep. from heated sap	0·25	6·8	135·9	—
268	350B	Material inactivated by lying in sap	0·35	4·7	130·2	0·89
297	531A	Inactivated by freezing	0·25	4·7	132·7	—
298	531B	Inactivated by freezing	0·25	4·7	131·1	—

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Partition Chromatography in the Study of Protein Constituents

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In the present paper we report new applications and developments of partition chromatography* [Martin & Synge, 1941*b*] in the study of amino-acids and peptides. We have further developed the technique, aiming first at the isolation and identification of protein constituents, both amino-acids and peptides, and secondly, at a reliable micro-analytical procedure for the determination of as many different amino-acids as possible in the same sample of a complete hydrolysate of a protein or peptide.

Here we describe the more technical aspects of the work and give a detailed account of the micro-method for monoamino-acids at its present stage of development, together with the results obtained with it on wool and gelatin hydrolysates. The two following papers illustrate further the application of our new methods to particular problems in protein chemistry. Throughout the present work we have employed, as previously, the acetyl derivatives of the amino-acids and peptides.

* We employ the term 'partition chromatography' at the suggestion of Dr E. Lester Smith, to distinguish it from the classical adsorption chromatography. Our earlier term 'liquid-liquid chromatography' was liable to confusion with the fractional elution procedure sometimes called 'liquid chromatography'.

EXPERIMENTAL

Preparation of silica gel for chromatography

Adsorption by the silica of the substances undergoing analysis could seriously alter their behaviour from that to be expected were their partition between the two liquid phases the only determining factor. There must be an adequate concentration of anti-adsorbent substance in the solvent employed [Martin & Synge, 1941*b*]. Silica gel prepared in different ways has very different adsorption properties, and the more adsorbent gels may require unsuitably high concentrations of alcohol before they are useful. Adsorption by the silica may manifest itself as 'tailing' of the solutes undergoing analysis, and results in imperfect separation. This is the behaviour to be expected for adsorption of the 'Freundlich' type, where, for low concentrations, the adsorption is relatively much greater than for high ones. 'Ageing' of the freshly precipitated gel under dilute acid before drying it is essential for the production of a suitable colour with methyl orange (MO), but if ageing is continued for much longer than prescribed, a serious increase in adsorbing power may result. Deviations from the precipitation procedure given below (order of addi-

tion, concentration, temperature, etc.) result in less satisfactory products. Thorough acidification during precipitation and ageing is essential. Otherwise, low recoveries of the first acid band moving down the column will result. This is presumably due to neutralization by retained Na^+ ions, and explains the low recovery of acetylphenylalanine noted previously.

Detailed procedure. The silica gel is prepared according to Martin & Synge [1941*b*, footnote]. We give here a fuller description.

Commercial water-glass (140° Tw.-Jos. Crosfield, Ltd., Warrington) is diluted to 3 vol. with distilled water containing a little MO. 10*N* HCl is added in a thin stream with vigorous stirring, addition being interrupted at intervals and stirring continued to get efficient mixing. The solution changes first slowly and then rapidly to a thick porridge and all but the smallest lumps are broken up by stirring. When the mixture is permanently acid to thymol blue, addition of HCl is stopped and the mixture is kept 3 hr. It is filtered on a Buchner funnel and washed with distilled water (approx. 2 l./250 g. dry gel) without allowing the precipitate to crack. The gel is then suspended in *N*/5 HCl and aged 2 days at room temperature. It is again filtered and washed in the same way with distilled water (approx. 5 l./250 g. dry gel) until the washings are free from MO. Finally, the gel is crumbled and dried at 110° in an air oven. With such a preparation the addition of 53 % w/w of indicator solution to the dry gel should be satisfactory with the butanol-chloroform or propanol-cyclohexane mixtures. The dry gel can be stored in a closed vessel for long periods without deterioration.

Indicators

MO has been employed throughout these experiments for indicating the presence of the acetyl derivatives. Most of the usual indicators of suitable *p*H range which we have studied are very readily extracted from the aqueous phase by the developing solvent. MO is not free from this defect, and is slowly removed by the solvent, particularly if the alcohol content of the latter is high. When this happens, the column becomes colourless from above downwards, and is useless for the visual separation of solutes whose bands move much slower than that of acetylglycine.

The anthocyanin series promises to yield suitable indicators. Of those tested, an aqueous solution (0·05 %) of pelargonin chloride (prepared from petals of the scarlet dahlia 'Coltness Gem' [cf. Willstätter & Mallison, 1915]) has proved useful. It is not readily 'leached' by solvent mixtures quite rich in alcohols; its sensitivity to acid is not (like that of MO) markedly depressed by alcohols; and it is free from N and S.

The presence of MO in the fractions obtained from the chromatograms has not interfered with the titration of the acetyl derivatives described below, but the indicator has sometimes been difficult to eliminate in the course of preparative work.

The 'background' colour of the MO in the chromatograms is greatly affected by the nature of the developing solvent, as well as by the silica, and may vary from bright pink to bright yellow. The solvents whose use we describe below all give usable orange background colours, although 30 % propanol-cyclohexane gives a rather yellow and insensitive column. Good lighting is essential for making the best 'cut' when the bands are not widely separated. In the absence of bright daylight we have used a 80 W B.T.H. fluorescent lamp with success.

Behaviour of amino-acid and peptide derivatives in different solvent systems

We have studied in detail three different solvent systems in which the acetyl derivatives of the mono-amino-acids and some of their peptides have suitable distributions, viz.: (1) chloroform-*n*-butanol-water, (2) cyclohexane-*n*-propanol-water, (3) ethyl acetate-water. Tables 1 and 2 give the band-rates of a number of those compounds in chromatograms employing these solvent systems. R has the same significance as before [Martin & Synge, 1941*b*], and may be related to the partition coefficient by the same formula [*loc. cit.*]. Since R depends on a number of variable factors, the values which we give must be regarded as a rough guide only. The ratios between these values for different compounds in the same column are, however, relatively constant.

Here and subsequently such an expression as 1 % butanol-chloroform refers to a solution in chloroform of butanol, 1 % v/v, subsequently saturated with water.

(1) *Acetamino-acids.* While in general the acetamino-acids with longer and less polar 'side-chains' move fastest on the chromatograms, there are some striking differences in their sequence in the different solvents. Thus while acetylphenylalanine travels faster than acetylleucine in chloroform-butanol-water, it is not separable from it in cyclohexane-propanol-water. Acetyltryptophan, acetylmethionine and acetylproline are also relatively retarded in the latter solvent. The same is generally true for this 'more polar' class of acetamino-acids in ethyl acetate-water as against chloroform-butanol-water. In ethyl acetate-water, however, *N*-acetyltyrosine is considerably accelerated.

In general, the less polar solvent (cyclohexane) discriminates more between these classes of compound than does the more polar solvent ($CHCl_3$). However, a single parameter such as 'polarity' probably cannot be employed for giving a complete

Table 1. *Band rates of acetamino-acids with various solvents*

(Figures are values of R)

Developing solvents	Butanol-chloroform 1%	Butanol-chloroform 3%	Butanol-chloroform 17%	Propanol-cyclohexane 5%	Propanol-cyclohexane 30%	Ethyl acetate
Acetylphenylalanine	0·5	0·9	Fast	0·3	Fast	Fast
Acetyl*nor*leucine	0·4	—	Fast	—	—	—
Acetylleucine / Acetyl*iso*leucine	0·3	0·6	Fast	0·3	Fast	Fast
Acetyltryptophan	0·3	0·6	Fast	0·15	Fast	—
Acetylvaline / Acetyl*nor*valine	0·15	0·3	Fast	0·15	Fast	0·9
Acetylmethionine	0·15	0·3	Fast	0·09	0·7	0·8
Acetylproline	0·15	0·3	Fast	0·04	0·3	0·2
α-Acetaminobutyric acid	0·07	—	—	—	—	0·5
Acetylalanine	0·025	0·04	0·35	0·04	0·3	0·2
N-Acetyltyrosine	0·02	0·04	0·7	—	0·6	Fast
Acetylglycine	Slow	Slow	0·15	Slow	0·15	0·1
Acetylaspartic acid / Acetylglutamic acid / *NN'*-Diacetylcystine / *NN'*-Diacetyllysine	Slow	Slow	0·1–0·2	—	—	—
N-Acetylhydroxyproline	Slow	Slow	0·07	Slow	0·1	0·06

theoretical description of such systems. Our experience suggests that new and useful solvent mixtures having specific effects with particular side-chains remain to be found.

We have not yet effected a satisfactory separation of leucine from *iso*leucine, or of valine from *nor*valine, and are now unable to confirm the separation of the former using 0·5 % butanol-chloroform previously reported [Martin & Synge, 1941*b*]. We have not observed any separation of an optically active substance from the corresponding racemic compound in the course of this work.

(2) *Acetylpeptides.* The evidence presented in Table 2 suggests that acetylpeptides travel in general at rates intermediate between those of the acetyl

Table 2. *Band rates of acetyldipeptides with various solvents*

(Figures are values of R)

Developing solvents	Butanol-chloroform 1%	Butanol-chloroform 17%	Propanol-cyclohexane 5%	Propanol-cyclohexane 30%	Ethyl acetate
Acetylleucylleucine	1·0	Fast	—	—	Fast
Acetylleucylglycine / Acetylglycylleucine	0·04	0·6	0·04	1·0	0·5
Acetylglycylglycine	Slow	0·04	—	—	0·06

derivatives of their constituent amino-acids, and this is confirmed by experiments on the products of the partial hydrolysis of gelatin [Gordon, Martin & Synge, 1943*b*].

The data of Tiselius [1942] on the behaviour of free amino-acids and peptides in adsorption chromatograms packed with carbon and developed with aqueous solutions show a considerable difference in sequence from that obtained with our systems. Use of the two methods in succession might lead to highly effective separations of peptides. However, before Tiselius's chromatograms can be used for the isolation of substances it will be necessary to eliminate 'tailing' of the bands, which the adsorption data given by Cheldelin & Williams [1942] lead us to expect [cf. Wachtel & Cassidy, 1942]. Tiselius's use of 'moving boundary' observation methods with a chromatogram continuously fed with the mixture to be investigated is not interfered with by 'tailing'—which, indeed, increases the sharpness of the front boundary of each solute. The disadvantages of this procedure for isolative work are manifest, only a small portion of the least adsorbed component being readily obtainable in a state of purity.

Preparation and properties of acetyl derivatives studied

The compounds referred to in Tables 1 and 2 were described by Synge [1939] with bibliography, except for the following. Compounds reported here for the first time are indicated by an asterisk. All M.P.'s are given uncorrected unless otherwise stated. N determinations were by the Kjeldahl procedure. C and H determinations were carried out for us by Dr G. Weiler, Oxford.

(1) *Acetyl-l-valine.* The earlier preparation made by acetylation of *l*(+)-valine ('*d*-valin' of Hofmann La Roche) proved on chromatographic investigation to be a mixture containing at least 15% of impurity, probably an acetylleucine isomer. This would account for the deviations of the analytical values from the theoretical recorded by Synge [1939].

Our present material was prepared by resolution of acetyl-*dl*-valine [cf. Synge, 1939] obtained by acetylation of a specimen of *dl*-valine which we had prepared by a malonic ester synthesis from *iso*propyl bromide [Marvel & du Vigneaud, 1931; Marvel, 1940]. The acetyl-*dl*-valine was resolved directly as its brucine salt. A number of intermediate stages that would have been necessary had we employed Fischer's [1906] resolution were thus avoided.

3 g. of acetyl-*dl*-valine and 7·5 g. of brucine were dissoved in a minimum of ethanol, and ether was added to turbidity. Slow crystallization resulted, which in later preparations could be accelerated by seeding and stirring. The crystalline brucine salt was filtered off, washed with ether, and recrystallized in the same way from ethanol-ether. The recrystallized product weighed 4·2 g. Brucine was removed from the salt by treatment with NaOH in aqueous solution at 0°. After filtration of the precipitated brucine, the solution was extracted with $CHCl_3$ to remove the part remaining in solution. Dilute H_2SO_4 equivalent to the NaOH employed was then added, the mixture was evaporated to dryness *in vacuo*, and the solid residue was thoroughly extracted with boiling ethanol. On evaporation of the combined alcoholic extracts, crystalline acetyl-*l*-valine resulted, which was recrystallized from water until its optical rotation and M.P. were constant. Yield 0·9 g., having M.P. 164°, $(\alpha)_D^{19°}$ +4·0° (ethanol, $c=2$). The preparation was chromatographically homogeneous. These physical data supersede those given for acetyl-*l*-valine by Synge [1939].

(2)* *Acetyl*-dl-nor*valine*. This was prepared from a specimen of *dl-nor*valine (manufactured by Fränkel and Landau, and kindly given to us by Prof. H. A. Krebs) in the way described by Synge [1939]. The product on recrystallization from water had constant M.P. 111–112·5°. It proved, however, on chromatographic investigation to contain an impurity (at least 15%), probably an acetylleucine isomer. This suggests the occurrence of mixed crystals. As the preparation was required for qualitative chromatographic study, the presence of this impurity was not an obstacle, and we have not yet purified the compound. The elementary composition of the preparation is in agreement with that expected from the chromatographic data. (Found: C, 53·5; H, 8·27; N, 8·67%. $C_7H_{13}O_3N$ requires: C, 52·8; H, 8·17; N, 8·8%. $C_8H_{15}O_3N$ requires: C, 55·5; H, 8·67; N, 8·1%.)

(3)* *NN'-Diacetyl*-dl-*lysine* [cf. Synge, 1939; Kolb & Toennies, 1942]. Synthetic *dl*-lysine was acetylated in aqueous solution with NaOH and acetic anhydride as usual. After acidification with H_2SO_4, the aqueous solution was extracted repeatedly with ethyl acetate. The syrupy residue from evaporation of the combined extracts crystallized after keeping for several weeks. The product was readily recrystallized from ethyl acetate, in which it is rather insoluble. M.P. 140–141°. (Found: C, 52·0; H, 7·62; N, 12·1%; acid equiv. wt. 232. $C_{10}H_{18}O_4N_2$ requires: C, 52·2; H, 7·82; N, 12·2%; acid equiv. wt. 230.)

(4)* *Acetyl*-l-*aspartic acid*. This was prepared from *l*-aspartic acid by acetylation and extraction from acid solution with ethyl acetate as for the previous compound. The product was syrupy.

(5) *Acetyl*-l-*tryptophan*. This was prepared according to du Vigneaud & Sealock [1932] from *l*-tryptophan. Our product had M.P. 185–6°, $(\alpha)_D^{21·5°}$ +27·6° (water containing 1 equiv. of NaOH, $c=1$), $(\alpha)_D^{19°}$ +26·0° (ethanol, $c=1·1$). Du Vigneaud & Sealock record M.P. 189–190° (corr.), $(\alpha)_D^{31°}$ +29° (water containing 1 equiv. of NaOH, $c=1$). From $CHCl_3$ the compound was found to crystallize with 1 mol. of solvent. This was lost only slowly in a vacuum desiccator at room temperature, but was readily removed by drying *in vacuo* at 100°. Under these conditions the specimen lost 30·2% of its weight. (Loss of wt. calc. for $C_{13}H_{14}O_3N_2.CHCl_3$ is 32·7%.)

(6) *Acetylglycyl*-dl-*leucine*. Prepared according to Steiger [1934] from glycyl-*dl*-leucine [cf. Fischer & Warburg, 1905]. M.P. 176–177°. Steiger records 178–180° (corr.). (Acid equiv. wt.: Found, 223. Calc. for $C_{10}H_{18}O_4N_2$, 230.)

(7) *Acetyl*-dl-*leucylglycine*. Prepared according to Steiger [1934] from *dl*-leucylglycine [cf. Fischer & Brunner, 1905]. When crystallized from acetone containing 5% water the compound crystallized with $\frac{2}{3}$ mol. of acetone, which could be removed by drying over P_2O_5 *in vacuo* at 100°. The undried compound had acid equiv. wt. 270, and lost 14·4% of its weight on drying. (Calc. for $C_{10}H_{18}O_4N_2$, $\frac{2}{3}$ (C_3H_6O): acid equiv. wt. 268·6; loss of weight on drying, 14·3%.) The dried material regained the lost weight in an atmosphere saturated with acetone vapour, but not in an atmosphere saturated with water vapour. The dried material had M.P. 178°. Steiger records M.P. 182–183·5° (corr.). He does not mention the occurrence of acetone of crystallization.

(8) *Acetylglycylglycine*. Prepared from glycylglycine [cf. Fischer & Fourneau, 1901] by Steiger's [1934] acetylation procedure. Recrystallized from ethanol, the product had M.P. 178–179°. Fischer & Otto [1903] record M.P. 184–186°. (Acid equiv. wt.: Found, 175. Calc. for $C_6H_{10}O_4N_2$, 174.)

(9)* *Acetyl*-dl-*leucyl*-dl-*leucine*. This was prepared from the racemic leucylleucine [cf. Fischer, 1904] subsequently named leucylleucine 'A' by Fischer & Koelker [1907] and shown by them to be the racemic compound involving *d*-leucyl-*l*-leucine as opposed to *d*-leucyl-*d*-leucine. We acetylated the dipeptide with acetic anhydride and excess NaOH in aqueous solution as usual, and on acidification of the reaction mixture the acetyl compound crystallized out in 71% yield. Recrystallization from ethyl acetate yielded a product having M.P. 200°. (Found: C, 58·6; H, 9·02; N, 9·4%; acid equiv. wt. 278. $C_{14}H_{26}O_4N_2$ requires: C, 58·7; H, 9·09; N, 9·8%; acid equiv. wt. 286.)

Micro-determination of monoamino-acids

The method of analysis to be described is a development of that given by Martin & Synge [1941*b*], and is based on the observations reported above. By the use of additional solvent mixtures it is now possible to determine the following amino-acids and groups of amino-acids in a protein hydrolysate, viz. phenylalanine, leucine + *iso*leucine, valine (+*nor*valine, if present), methionine, proline, alanine, and tyrosine. Tryptophan may also be determined semi-quantitatively if it survives the treatment employed for hydrolysing the specimen [cf. Gordon *et al.* 1943*a*]. Also in certain special cases [cf. Gordon *et al.* 1943 *a*, *b*] glycine has been determined semi-quantitatively by this procedure. A satisfactory determination of lysine as its diacetyl derivative could be developed on these lines for use with basic amino-acid fractions prepared by electrodialysis or

precipitation with phosphotungstic acid. The need for a direct determination of this amino-acid has long been felt.

This extension of the range of the method is due to two novel features:

(1) After the faster moving constituents of the mixture undergoing analysis have left the column, some of the remaining slow-moving constituents are accelerated and run out by the use of a higher percentage of alcohol in the developing solvent. This has brought alanine, tyrosine, etc., within the scope of the method.

(2) By subjecting each fraction obtained from the butanol-chloroform chromatogram to further fractionation on a propanol-cyclohexane chromatogram, it is possible first to separate the (proline + valine + methionine) fraction into its three constituents, and secondly to remove a number of impurities (probably decomposition products resulting from prolonged treatment of the mixture with hot acid) which affect significantly the accuracy of analyses by the procedure originally described.

It is sometimes advantageous to change the order in which the chromatograms are used [cf. Gordon *et al.* 1943*a*], or to adopt different solvent mixtures. The present scheme, however, is likely to be a useful and routine approach where little is known about the constituents of the mixture for analysis. With the method described here, the amino-acids in question may in the first instance be determined on the hydrolysate of a single sample of protein with an accuracy of $\pm 5\%$. It may then be possible to devise control experiments [cf. Gordon *et al.* 1943*a*] for increasing the accuracy of the determination.

These analytical results are not affected by the degree of racemization of the amino-acids. As pointed out by Moore, Stein & Bergmann [1942], this is an inherent defect of their 'solubility product' techniques.

Our new procedure yields results of accuracy comparable with that of the better routine amino-acid determinations in current use, and furthermore covers a group of amino-acids for which analytical methods have in the past been inadequate. Since, in addition, it is on the microchemical scale (about seven different components being determinable on a 25 mg. sample of protein), it may help to satisfy a long-felt need.

Preparation of material for chromatography. Further work on the acetylation and extraction procedure described by Martin & Synge [1941*b*] has led us to introduce some improvements and simplifications. The method at present in use is as follows. The HCl hydrolysate to be acetylated (corresponding to about 25 mg. of protein) is evaporated *in vacuo* to dryness in a 100 ml. Erlenmayer flask. 2 ml. of 2*N* NaOH are added and then 0·4 ml. of 6*N* NaOH. The solution is then cooled to 0°, and 0·2 ml. of acetic anhydride is immediately introduced, after which the flask is shaken and again cooled. Four further additions of 2 ml. of 2*N* NaOH, followed by 0·2 ml. of acetic anhydride, are made at 2 min. intervals and are accompanied by cooling and shaking. Thymolphthalein (as an external indicator) is used to ensure that the solution remains alkaline throughout. The solution is then allowed to stand for 10 min., care being taken to see that it remains alkaline. If this is not done anomalous bands appear which are probably due to *ON*-diacetyl hydroxyamino-acids which are formed if insufficient NaOH is present [cf. du Vigneaud & Meyer, 1932]. The *O*-acetyl groups are readily saponified by alkali in the cold. At the end of the 10 min. period, the solution is made acid (red) to thymol blue by the addition of 10*N* H_2SO_4. Next it is transferred to a 60 ml. separating funnel and extracted with five successive 50 ml. portions of 17% butanol-chloroform. The extracting solvent is used to rinse out the flask after transferring the mixture to the separating funnel. The $CHCl_3$ extracts are filtered through a dry filter paper (Whatman no. 2) into a Claisen flask from which the solvent is subsequently evaporated *in vacuo*, the temperature being allowed to rise to 100°. Finally, the extracted acetamino-acids are transferred in ethanol to a small flask, the ethanol is removed *in vacuo*, and the residue kept overnight in a vacuum desiccator over soda-lime and H_2SO_4.

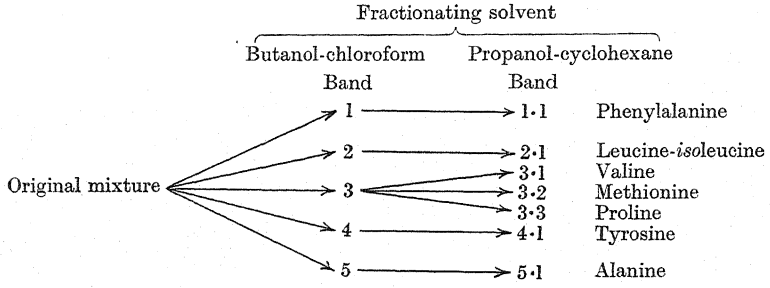
Chromatographic procedure. 3 g. of silica gel (prepared as described above) are saturated with methyl orange solution and suspended in 3% butanol-chloroform. This is introduced into a chromatogram tube having an internal diameter of 1 cm. [Martin & Synge, 1941*b*]. When the column is ready, as much as possible of the acetylated mixture is transferred to it by repeated (hot) extraction with small portions (1 ml. or less) of the solvent mixture. Each portion is allowed to drain into the gel before the next is added. After about five successive extractions, the flask containing the unextracted material is put aside for the second stage of the analysis, and the chromatogram tube is filled with the solvent mixture for development (3% butanol-chloroform).

The bands corresponding to phenylalanine (1), (leucine + *iso*leucine) (2), and (proline + valine + methionine) (3), are allowed to run out and are collected separately in pyrex flasks. The surface of the solvent is then allowed to sink until it is level with the top of the silica gel. At this point the acetylated material remaining in the flask, previously dissolved by boiling with about 1 ml. of 17% butanol-chloroform, is run on to the column. This is allowed to sink in, and after the flask has been washed out three times as before, development of the chromatogram is continued with the new solvent. This causes the bands corresponding to tyrosine and alanine, which have up to now been moving at nearly the same rate (ahead of the glycine, cystine, lysine, glutamic acid, etc.), to move faster, and at the same time draw apart from one another, the tyrosine band (4) running approximately twice as fast as the alanine band (5). These fractions are allowed to run out, and are collected separately.

Fractions 1–5 are freed from butanol-chloroform by evaporation on the water-bath *in vacuo*. Frac-

tions 1 and 2 are further analysed exactly as before, on columns containing 2 g. of silica, but using 5% propanol-cyclohexane instead of butanol-chloroform. The main band from each chromatogram is run out and collected (1·1 and 2·1, Table 3) free from any minor contaminants moving at different rates. Fraction 3 is similarly fractionated with the same solvent mixture on a rather longer column employing 3 g. of silica. Here the first band to be collected corresponds to valine (3·1). When this has run out, the solvent mixture is changed to 30% propanol-cyclohexane, and the bands corresponding to methionine (3·2) and proline (3·3) are collected separately. Fractions 4 and 5 are fractionated on columns containing 2 g. of silica and developed with 30% propanol-cyclohexane. These result in the main fractions 4·1 and 5·1. Table 3 illustrates this fractionation.

Table 3. *Scheme of fractionation*



Titration of resulting fractions. The seven final fractions are freed from solvent by evaporation *in vacuo* on the water-bath; the residue in each flask is dissolved in 3 ml. of water (by heating if necessary) and titrated against $N/100$ $Ba(OH)_2$ using bromthymol blue as internal indicator. This is used in preference to phenolphthalein because the indicator blank is smaller and it is not affected by room concentration of CO_2; further, it gives with *N*-acetyltyrosine accurate titrations of the —COOH group only. With phenolphthalein the phenolic group of this compound titrates partially, giving a high result with an unsatisfactory end-point.

For all the amino-acids determined at present except tryptophan, 1 ml. of $N/100$ $Ba(OH)_2$ corresponds to 0·14 mg. of N; for tryptophan it corresponds to 0·28 mg. of N.

Control experiments on procedure. The mixture of amino-acids and their acetyl derivatives shown in Table 4, which simulates in amino-acid composition approximately 25 mg. of wool, was refluxed in an excess of 6*N* HCl for 24 hr., and was then analysed as above.

In this experiment 3% butanol-chloroform was used for the initial fractionation because it causes more rapid movement of the bands, even though it gives rather poor separations between bands 1, 2 and 3. Thus the low recovery of phenylalanine and the excess of leucine no doubt result from the collection of some of the former material with the leucine band. Similarly, some of the leucine was collected in the proline-valine-methionine band, but this does not affect the accuracy of the analysis because, although inseparable from phenylalanine, leucine can easily be separated from proline, valine and methionine on the propanol-cyclohexane column. As previously reported [Martin & Synge, 1941*b*], good separations of bands 1, 2 and 3 can be obtained by using 1% butanol-chloroform, and unless a longer column is used, this solvent is recommended for development until bands 1 and 2 have been run out of the column.

Table 4. *Control analysis on an amino-acid mixture*

	Taken (mg. N)	Found (mg. N)	Recovery (%)
Acetyl-*dl*-phenylalanine	0·0954	0·088	92
Acetyl-*d*-leucine	0·2420	0·310	104
Acetyl-*d*-*iso*leucine	0·0566		
Acetyl-*dl*-valine	0·1637	0·156	95
l-Methionine	0·0226	0·021	93
Acetyl-*l*-proline hydrate	0·1799	0·189	105
Acetyl-*dl*-alanine	0·1700	0·165	97
l-Tyrosine	0·0795	0·082	103
Acetyl-*l*-tryptophan	0·0376	—	—
Glycine	0·5330	—	—
l-Cystine	0·3455	—	—
l-Glutamic acid	0·2555	—	—
l-Aspartic acid	0·1295	—	—
dl-Serine	0·2478	—	—
dl-Threonine	0·1928	—	—
l-Arginine monohydrochloride	0·9280	—	—
dl-Lysine dihydrochloride	0·1330	—	—
l-Histidine monohydrochloride	0·0417	—	—
Ammonium sulphate	0·3415	—	—
Total	4·1956		

No tryptophan survived the conditions to which the mixture was subjected—had any done so it would have been detected during the further fractionation of fraction 2 with propanol-cyclohexane. The work reported in our paper on gramicidin

[Gordon *et al.* 1943*a*] shows that substantial recoveries of acetyltryptophan result when tryptophan is present, with simpler amino-acids, in large amount, and when the hydrolysis is conducted with exclusion of air. Even here, however, some destruction of tryptophan occurs.

In the control experiment here reported, the following 'artefact bands' were noted during refractionation of the materials from the first column with propanol-cyclohexane. All were present in small amount.

Fraction 1. (*a*) A band travelling twice as fast as the phenylalanine band. (*b*) A band remaining near the top of the chromatogram.

Fraction 3. (*a*) A band travelling between the methionine and proline bands. (*b*) A band travelling just ahead of the proline band, but so close that its adequate elimination proved difficult.

Fraction 4. (*a*) A band moving at about half the rate of the tyrosine band.

It seems possible from the high value of the tyrosine recovery, that a contaminant not separable from it by this procedure is present. Although none of these artefact contaminants is present in very large amount, they render quite inaccurate the single fractionation procedure originally given by Martin & Synge [1941*b*]. Moreover, as they are probably formed by alteration of the original components of the mixture, this may explain the lower accuracy of the present determinations where a known complicated mixture was heated with acid in the open, as compared with those reported by Gordon *et al.* [1943*b*] for a much simpler known mixture heated *in vacuo*. These considerations are important and introduce a factor of uncertainty, stressed by Moore *et al.* [1942], into the process of relating the results of analysis of a protein hydrolysate to the amino-acid composition of the intact protein.

Analysis of wool and gelatin

Wool (Merino 64's) and gelatin (from the same batch of cow-hide gelatin studied by Gordon *et al.* 1943*b*) were subjected to analysis by the procedure given above. The results of the analysis (uncorrected) are shown in Table 5, together with previous data on wool. The analysis of gelatin is correlated with the data of other workers in an accompanying paper [Gordon *et al.* 1943*b*].

Before acetylation the wool hydrolysate had been extracted with $CHCl_3$ to remove non-amino-acid constituents [cf. Martin & Synge, 1941*a*]. The artefact contaminants noted in the control analysis were all present also in the wool hydrolysate, but one additional artefact was noted, which appeared as a slow-moving band in fraction 2.

In the gelatin hydrolysate, contaminants 1(*a*), 3(*a*), 3(*b*) and 4(*a*) were absent, but two faint bands (one ahead of and one behind the leucine band) were noted during the fractionation of band 2. A faint band running at about half the rate of the proline band was noted in the fractionation of band 3. Some of these differences may be attributed to the absence of tryptophan from gelatin; the absence of cystine is probably also of significance.

The most striking feature of the analysis of wool is the phenylalanine value, which has been much lowered by the elimination of 'artefacts'. The possibility that aromatic constituents as yet unknown are present is suggested by Block's [1939] higher value, obtained by the not very specific colorimetric procedure.

Otherwise the results are in substantial agreement with those previously recorded [cf. Martin & Synge, 1941*a*, for bibliography]. The value for alanine is the first to be reported for wool since Abderhalden & Voitinovici's [1907] ester analysis, and is in substantial agreement with their figure.

SUMMARY

1. Technical and theoretical aspects of partition chromatography are discussed in relation to the separation of amino-acids and peptides in the form of their acetyl derivatives.

2. New data are presented on the behaviour of a number of these compounds in different solvent systems.

3. A micro-analytical procedure is described for the simultaneous determination of phenylalanine,

Table 5. *Analyses of wool and gelatin*

(N as % of total N)

	Gelatin	*Wool*			
	Present detn.	Present detn.	Martin & Synge [1941*b*]	Other detns.	
Phenylalanine	0·4	0·8	1·9	1·9	
Leucine-*iso*leucine	3·9	6·2	7·4	7·2	
Valine	1·7	3·9		3·4	Martin & Synge [1941*a*]
Methionine	0·3	0·2	8·2	0·4	
Proline	9·6	5·2		4·9	
Tyrosine	? Trace	2·8	—	2·1	Marston [1928]
				2·3	Block [1939]
Alanine	8·3	3·9	—	4·1	Abderhalden & Voitinovici [1907]

leucine isomers, valine, methionine, proline, alanine and tyrosine in the hydrolysate of a single sample of protein.

4. Determinations of these constituents of wool and cow-hide gelatin are reported.

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The Amino-Acid Composition of Gramicidin

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The bactericidal substance gramicidin, isolated by Dubos *et al.* from cultures of various bacteria [for bibliography cf. Dubos & Hotchkiss, 1942] is of polypeptide character, yielding on acid hydrolysis a mixture of amino-acids, some of which possess the 'unnatural' *d*-configuration [Hotchkiss, 1941; Lipmann, Hotchkiss & Dubos, 1941; Christensen, Edwards & Piersma, 1941].

We have subjected acid hydrolysates of gramicidin to analysis by our new techniques [Gordon, Martin & Synge, 1943*a*] and have been able to confirm and extend most of the data of these authors.

Taken in conjunction with the analytical results of Hotchkiss [1941], our analyses suggest that the gramicidin molecule contains 24 amino-acid residues or some integral multiple thereof. These are made up as shown in Table 1.

The presence of leucine and tryptophan residues in gramicidin has already been established by isolation and characterization [Hotchkiss, 1941; Christensen *et al.* 1941]. Christensen *et al.* also claim to have isolated alanine through its dioxpyridate and phosphotungstate, although no experimental details are given. They obtained a negative result for glycine, using the colorimetric method of Patton [1934]. We have established the occurrence in the hydrolysate of alanine, valine and glycine by isolation and characterization as the acetyl derivatives, and have confirmed in the same way the presence of leucine and tryptophan.

Table 1. *Suggested composition of minimum molecule of gramicidin*

	No. of residues	No. of atoms N
Leucine	6	6
Tryptophan	6	12
Valine	5	5
Alanine	3	3
Glycine	2	2
Hydroxyamino compound	2	2
Total	24	30

Our technique was not suitable for the isolation of the hydroxyamino compound [Hotchkiss, 1941]. We have, however, established by control experiments (see Experimental Section) that serine is

absent from gramicidin hydrolysates. Our methods would probably also reveal higher aliphatic hydroxyamino-acids, and on the evidence presented by Hotchkiss, *iso*serine would seem to be the substance whose properties agree best with what is known at present of this constituent of gramicidin.

Although our isolative procedure leads to slight racemization [cf. Martin & Synge, 1941], we have been able to confirm that the leucine of the hydrolysate is largely of the *d*-configuration, while the tryptophan has the *l*-configuration. The alanine is also largely in the *l*-form. We find, however, that the valine isolated is racemic, and since this method of isolation enabled us to prepare from wool [Martin & Synge, 1941] and from gelatin [Gordon *et al.* 1943*c*] acetylvaline retaining a large part of its optical activity, we conclude either that the valine residues of gramicidin are very readily racemized during hydrolysis, or that both *d*- and *l*-valine residues co-exist in the gramicidin molecule. It is impossible at present to choose between these alternatives. We have previously noted a similar phenomenon, viz. the occurrence of racemic phenylalanine in wool hydrolysates, and the possibility that selective racemization occurs during acid hydrolysis has been discussed in that connexion [Martin & Synge, 1941] (cf. Gordon *et al.* [1943*a*] for isolation of optically active acetylphenylalanine by a similar procedure).

Since glycine does not react with the *d*-amino-acid oxidase [Krebs, 1935], the α-amino-acids oxidized by the latter [Lipmann *et al.* 1941] must consist largely of leucine and of half the valine residues. These amount to 38·6 % of the total α-amino-acid residues in the postulated molecule, in fair agreement with the figure 43–46 % determined by Lipmann *et al.*

In Table 2 we correlate our experimental data with those of Hotchkiss [1941] and with the values calculated for the postulated minimum molecule containing 30 atoms of nitrogen.

Table 2. *Constituents of gramicidin hydrolysate*

(N as % of total N)

	Present detn.	Hotchkiss [1941]	Calc.
Leucine	20·2	16·1*	20·0
Tryptophan	40–45	37·3	40·0
Valine	16·6	—	16·7
Alanine	10·1	(25–35)	10·0
Glycine	5·3–6·6	—	6·7
Hydroxyamino N + NH_3-N	—	6·0	6·7
—OH groups (before hydrolysis) (equivs. as N)	—	7·3	6·7
α-Amino N (ninhydrin)	—	72·6	73·3
Amino-N (Van Slyke)	—	80·4	80·0
Basic groups by titration (equivs. as N)	—	80·5	80·0
Acidic groups by titration (equivs. as N)	—	84·7	80·0

* By isolation.

Our values for tryptophan and glycine are semi-quantitative only, for reasons discussed in the Experimental Section.

The method of Kendall & Friedemann [1930] used by Hotchkiss for determining alanine gives seriously high values in the presence of valine and leucine.

We have added Hotchkiss's figure for NH_3 in the hydrolysate to his determined hydroxyamino N, since the hydroxyamino compound seems a probable source of the NH_3 liberated during treatment with hot acid [cf. Hotchkiss, 1941; Gordon, Martin & Synge, 1941].

There is reasonable agreement between the experimental data and the figures calculated for the molecule postulated in Table 1.

This minimum molecule deduced by stoichiometric reasoning has the molecular weight 2790, assuming that (i) the unknown hydroxyamino compound is *iso*serine, and (ii) the gramicidin molecule is a cyclopeptide of 24 residues, formed from the free amino-acids by elimination of 24 molecules of water.

This value for the molecular weight of gramicidin is in fair agreement with that found by Tishler, Stokes, Trenner & Conn [1941] by isothermal distillation in methanol, namely, 3100, and also with the value 2500–4500 found by the same authors by diffusion measurements in *n*-butanol. The cryoscopic determinations of these authors and of Hotchkiss [1941] gave lower values. The cryoscopic data of Tishler *et al.* for gramicidin in cyclohexanol showed apparent lowering of the molecular weight at low gramicidin concentrations. If this were due to a reversible dissociation of 'chemically bound' water from the gramicidin molecule, i.e. to water which is not removable by the usual drying procedures, but which is capable of equilibrium dissociation under conditions where it could be substituted by molecules of solvent, the results would be readily explicable. Such dissociated water, while affecting the cryoscopic data, would distil with the methanol in the isothermal distillation experiment, and thus escape detection. (Tishler *et al.* [1941] do not describe their drying procedure in detail.) If this were so the cryoscopic behaviour of gramicidin in cyclohexanol would be profoundly modified by drying it 'azeotropically' in dilute solution in cyclohexanol or similar solvent. Tishler *et al.* [1941] mention that gramicidin is hygroscopic. Abderhalden & Gohdes [1931] have described the binding of water by alanine peptides.

In the absence of further data it seems reasonable to conclude that the minimum molecular weight deduced for gramicidin by stoichiometric reasoning is the true molecular weight, and that we are dealing with a 24-cyclopeptide.

The equivalent weight given by Tishler *et al.* [1941] for gramicidin in combination with flavianic

acid (2986, 3036 ± 300) is in substantial agreement with the molecule postulated.

Assuming that the molecule contains two residues of *iso*serine and no chemically bound water, and that it is a cyclopeptide without terminal —NH_2 or —COOH groups, the molecular formula can be deduced, namely, $C_{146}H_{202}O_{26}N_{30}$, giving a calculated elementary composition of C, 62·8; H, 7·25; N, 15·05%. Hotchkiss & Dubos [1941] record C, 62·7; H, 7·59; N, 14·8%. Tishler *et al.* [1941] agree about the C and N content, but find H, 7·36–7·41%.

In the absence of information on the exact nature of the hydroxyamino compound and in view of possible 'chemically bound' water, one can only say that the composition postulated for gramicidin is not incompatible with its determined elementary composition.

The stoichiometric data obtained in the present study are in striking agreement with the Bergmann-Niemann hypothesis on protein structure, in that the total number of residues in the molecule, and the number of each species of residue except valine, are expressible in the form $2^m \times 3^n$, where m and n are integers. Even with the valine residues, in view of their unascertained optical character, it would be possible to postulate three *d*-residues and two *l*-residues or *vice versa* as independent components. Alternatively, gramicidin may not be a homogeneous substance but a 'molecular compound' of two or more similar, but distinct, peptides crystallizing together in definite proportions. In the Experimental Section of this paper we present evidence that substances made up of some of the same amino-acids as are in gramicidin, but present in different proportions, are to be found with it in tyrothricin.

As pointed out by us [Gordon *et al.* 1941], stoichiometric data do not justify conclusions about the relative positions of the various residues in the molecule, and we hope, should material be available, to throw light on the arrangement of the residues by a study of partial hydrolysis products [cf. Gordon *et al.* 1943*b*].

Gramicidin is the first naturally occurring peptide-like substance of this degree of complexity which is probably homogeneous, and for which the amino-acid composition has been ascertained with reasonable accuracy and completeness. Of the protamines, which appear to have similar molecular weights [cf. Svedberg & Pedersen, 1940], clupein, which has been most closely investigated, has been shown to be a mixture of substances fractionable by solvent precipitation of their methyl esters [Felix & Dirr, 1929; Felix & Mager, 1937]. Although it is certain that two-thirds of the residues of clupein are arginine residues, technical difficulties have in the past prevented accurate analysis of the monoamino-acids present. The hormone secretin appears to be a peptide of similar molecular weight [cf. Ågren, 1939]. Niemann [1939] has shown that, according to Ågren's analyses, some of its amino-acids occur in the proportions postulated by himself and Bergmann.

Apart from tyrocidine, which is found with gramicidin, few naturally occurring polypeptides of this order of molecular size have been accurately characterized. Systematic search will probably reveal more of them, and they may prove, as Kossel [1928] hoped, a valuable bridge between the very complicated proteins and their simple hydrolysis products. The study of such compounds will be particularly fruitful when they display physiological activity. The study of the partial hydrolysis products [cf. Gordon *et al.* 1943*b*] is likely at the present stage to give less equivocal results as to structural sequences in the molecule than the study of proteins containing hundreds of amino-acid residues. Also some of the partial hydrolysis products may retain physiological activity, and thereby throw light on its nature.

Dubos & Hotchkiss [1942] have reviewed biological aspects of the anti-bacterial action of gramicidin. The present work confirms that gramicidin is very unlike the average protein in amino-acid composition—notably in the absence of basic and acidic constituents and in the high proportion of tryptophan, leucine and valine. Lipmann *et al.* [1941] have discussed the possibility that the *d*-amino-acid residues play a part in its antibacterial action. Possibly the as yet unknown hydroxyamino compound has a special role, or the valine and leucine residues may have special inhibitory effects such as were noticed by Gladstone [1939] with these amino-acids in the free form in the nutrition of *Bacillus anthracis*. Unfortunately, Gladstone does not report experiments employing the *d*-isomers in the absence of the *l*-isomers. Perhaps, however, larger and more specific structural features of the molecule are concerned. Gramicidin might act in any of these ways if chemotherapeutic action depends on competitive inhibition of metabolic processes. Further study of its chemical structure along the above lines would seem to be a promising approach to this problem.

EXPERIMENTAL

Preparation of material for analysis—question of homogeneity

All samples of gramicidin were made by us from a Wallerstein preparation of tyrothricin. Four separate specimens were studied, the preparation of which we describe here. Before analysis all samples were dried to constant weight over H_2SO_4 and soda-lime in a vacuum desiccator at room temperature. N determinations were made by the Kjeldahl procedure after pre-treatment with red P and HI as recommended by Hotchkiss & Dubos [1941]. In our hands this procedure gave theoretical N values with acetyl-

tryptophan. Hotchkiss & Dubos record the N content of gramicidin as 14·8% and give the following optical data: $(\alpha)_D^{25°}$ +2·5° (ethanol, c=1·5), $(\alpha)_D^{25°}$ +5° (95% ethanol, c=0·4).

Preparation A. 4 g. of tyrothricin were worked up by the method of Hotchkiss & Dubos [1941]; the crude crystalline material was recrystallized once from acetone. After washing with ether the product weighed 117 mg. The whole preparation was hydrolysed for qualitative investigation of the amino-acids.

Preparation B. 1 g. of tyrothricin was worked up in the same way, the crude product was recrystallized thrice from acetone and washed with ether each time. Yield 19·9 mg. M.P. 226–227° (uncorr.). (Found: N, 14·6%.)

Preparation C was made from the combined mother liquors from the crystallization of preparations A and B, using the ethanol-ether precipitation of Hotchkiss & Dubos for preliminary purification. The first crop of crystals had $(\alpha)_D^{20°}$ −8·8° (ethanol, c=0·7). On recrystallization from acetone the product had $(\alpha)_D^{20°}$ −4·1° (ethanol, c=1·2). A further recrystallization yielded the final product, 18·5 mg., having $(\alpha)_D^{18·5°}$ +1·5° (ethanol, c=0·6). (Found: N, 13·0%.) In this, and the following preparation, washing of the crystals with ether was omitted, and this may have been responsible for the presence of the fatty material, which appeared as a separated layer after hydrolysis with acid. This fatty contaminant is presumably responsible for the lowered N content of preparations C and D.

Preparation D. This was made from the mother liquors from the recrystallization of preparation C. It did not readily yield crystals even after treatment with ethanol-ether. A purification by partition chromatography was attempted. It was found that gramicidin could be detected visually very readily owing to its fluorescence (presumably due to the tryptophan residues) on exposure to ultraviolet light. Trial experiments with silica gel chromatograms (using no indicator) showed that gramicidin can be made to move as a band. In the solvent system methanol-cyclohexane (the more polar phase being stationary, and absorbed in the gel), gramicidin scarcely moved at all on development. In aqueous systems, using as developing solvents 30% *n*-propanol-cyclohexane, 30% *iso*propanol-cyclohexane and 15% ethanol-cyclohexane, gramicidin moved as a band having R = >1, 0·5 and 0·2 respectively (same terminology as in previous paper [Gordon *et al.* 1943*a*]). The main preparation was then carried out on about 65 mg. of crude material, using 30% *iso*propanol-cyclohexane as the developing solvent. This caused the separation of three fluorescent fractions from the main band. One of these moved at R>1 (presumably fatty components), while the other two moved more slowly than the main fraction. This main fraction was run out of the column and collected separately; after evaporation of the solvent *in vacuo*, the preparation was taken up in ethanol and filtered to free it from silica which had escaped with it from the column. The ethanol was removed *in vacuo*, and the product was then allowed to crystallize from acetone. Crystallization was much slower than in previous preparations, taking 24 hr. for completion. The product, weighing 35 mg., had $(\alpha)_D^{20°}$ +3° (ethanol, c=1·1) and on recrystallization (an equally slow process) 18·3 mg. of the final product was obtained having $(\alpha)_D^{20°}$ +2° (ethanol, c=0·6). (Found: N, 12·9%.) Like preparation C, this material yielded a fatty layer on acid hydrolysis.

It seems therefore that some of the fatty material is so closely associated with the gramicidin that it resists chromatographic separation. The chromatographic purification, though eliminating the laevorotatory material noticed in the course of preparation C, does not give a really pure product, since, in addition to its slow crystallization, preparation D differs significantly in amino-acid composition from preparations B and C, having a lower content of valine and tryptophan, and a higher content of glycine, alanine and leucine than they have. This trend in composition of material obtained by working up the mother liquors (already noticeable in preparation C—cf. Table 6) suggests that, in addition to gramicidin, tyrothricin contains other components made up of some of the same amino-acids as the latter. In the analysis of none of these preparations have any qualitative differences in the amino-acids present been noted.

Although the chromatographic procedure used with preparation D did not lead to any dramatic purification, it seems likely that chromatographic methods may be of value for the quantitative extraction of gramicidin from tyrothricin preparations. The results mentioned here justify further study. From the data on simpler compounds given by Gordon *et al.* [1943*a*], gramicidin appears to distribute itself between solvent phases in a manner not inconsistent with its amino-acid composition.

Our overall yields of material judged to be rich in gramicidin have never amounted to more than 5% of the 5 g. of tyrothricin which we had at our disposal. This is rather less than the yields mentioned by Hotchkiss & Dubos [1941].

Antibacterial activity of the preparations

Dr F. C. Happold of Leeds University has kindly carried out bacterial growth inhibition tests [cf. Dubos & Cattaneo, 1939] on our preparations B, C and D, and reports:

'The samples of gramicidin have been tested for bactericidal or bacteriostatic action in tubes of beef infusion peptone broth (5 ml.). A 6 hr. culture of *Staphylococcus aureus* was inoculated into the experimental tubes and growth observed at 4, 5 and 6 hr. after inoculation. At 6 hr. a loop of each culture was spread over nutrient agar to give an additional comparison of the degree of growth. The gramicidin used was 200 μg./ml. in 2% ethanol and formed a stable suspension. A 1/10 dilution of this was also made in sterile distilled water so that when 0·2, 0·1 and 0·05 ml. of each of these two suspensions were added to the tubes of broth they gave concentrations of 40, 20, 10, 4, 2 and 1 μg./5 ml. medium.

'Preparations C and D were tested with a culture which was less rapidly viable than the one used with B. The result was even more dramatic. The

Table 3. *Antibacterial activity of gramicidin*

Preparation B	Growth at 4 hr.	5 hr.	6 hr.
Peptone infusion broth	+ + +	+ + + +	+ + + +
+ 0·2 ml. 2% ethanol	+ +	+ + + +	+ + + +
+ 1 μg. gramicidin	+	+ + +	+ + +
2 ,,	Tr.	+ +	+ +
4 ,,	Nil	?	+
10 ,,	Nil	Nil	? Tr.
20 ,,	Nil	Nil	Nil
40 ,,	Nil	Nil	Nil

alcohol controls and the infusion broth tube alone showed signs of growth at 6 or at 24 hr. When a loop of each tube was spread on agar all the gramicidin tubes were found to be obviously sterile.'

Analytical procedure

Following Hotchkiss [1941] we hydrolysed with HCl in aqueous acetic acid excluding air. After evaporation from alcoholic solution in a vacuum desiccator over H_2SO_4 and soda-lime, all our preparations (in Hysil test-tubes) were dissolved in 0·8 ml. of glacial acetic acid. 3·0 ml. of 6*N* HCl (which caused some precipitation) was added, the tube was evacuated, sealed, and heated for 24 hr. in a bath of refluxing 6*N* HCl. The precipitated material dissolved during the first ½ hr. of heating.

The hydrolysate was prepared for chromatography as described by Gordon *et al.* [1943*a*]. A preliminary investigation, using the analytical technique described there, revealed that a shortened procedure could be used for separating the acetyl derivatives. This is illustrated in Table 4.

Characterization of gramicidin constituents

Preparation A (117 mg.) was fractionated according to the procedure described above with columns of a suitably increased diameter. The resulting fractions were worked up and characterized as described below. All M.P.'s are uncorrected unless otherwise stated. N determinations were made by the Kjeldahl procedure on samples of material in solution, which samples had been titrated with *N*/100 $Ba(OH)_2$ to determine the acid equivalent weights, using the N-free indicator phenolphthalein. For the N determination on acetyltryptophan, the titration mixture was evaporated to dryness *in vacuo* and treated with red P and HI before incineration, as in the N determinations on gramicidin. C, H determinations were carried out by Dr G. Weiler, Oxford.

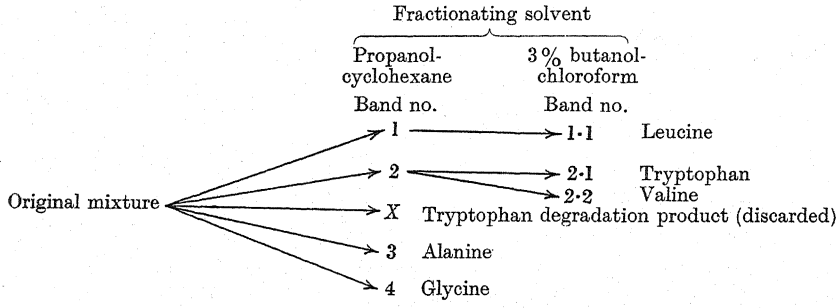
(1·1) *Acetyl*-d-*leucine.* Successive recrystallizations from *n*-propanol-cyclohexane and from water yielded 20 mg. of product having M.P. 183° (not depressed on admixture of authentic acetyl-*d*-leucine, but depressed on admixture of acetyl-*l*-leucine), $(\alpha)_D^{19°}$ +17·2° (ethanol, *c* = 0·7). Synge [1939] records for acetyl-*d*-leucine M.P. 186–188°, $(\alpha)_D^{22°}$ + 23·2° (ethanol, *c* = 3·7). (Found: C, 55·1; H, 8·61; N, 8·0%; acid equiv. wt. 174. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%; acid equiv. wt. 173.)

(2·1) *Acetyl*-l-*tryptophan.* Successive recrystallizations from chloroform-petrol and from water yielded 16 mg. of product having M.P. 178° (not depressed on admixture of authentic acetyl-*l*-tryptophan), $(\alpha)_D^{20°}$ +23·6° (ethanol, *c* = 0·5). Du Vigneaud & Sealock [1932] record M.P. 189–190° (corr.), and Gordon *et al.* [1943*a*] record $(\alpha)_D^{19°}$ +26·0° (ethanol, *c* = 1·1). (Found: C, 62·4; H, 5·68%. $C_{13}H_{14}O_3N_2$ requires: C, 63·3; H, 5·69%.) The material, recrystallized from $CHCl_3$, was further analysed as the molecular compound with $CHCl_3$ [cf. Gordon *et al.* 1943*a*]. (Found: N, 7·4%; acid equiv. wt. 358. Calc. for $C_{13}H_{14}O_3N_2.CHCl_3$: N, 7·7%; acid equiv. wt. 365·5.)

(2·2) *Acetyl*-dl-*valine.* A determination of optical rotation on the whole fraction (44 mg.) gave $(\alpha)_D^{20·5°}0 \pm 0·3°$ (ethanol, *c* = 1·5). Gordon *et al.* [1943*a*] give for acetyl-*l*-valine $(\alpha)_D^{19°}$ +4·0° (ethanol, *c* = 2). On this basis the present material contains >92·5% of the racemic compound.

Two recrystallizations from $CHCl_3$ yielded a product having M.P. 145° (not depressed on admixture of authentic acetyl-*dl*-valine for which Synge [1939] records M.P. 144–146°). Acetyl-*dl-nor*valine [cf. Gordon *et al.* 1943*a*] appears

Table 4. *Scheme of fractionation*



In the propanol-cyclohexane column fraction 1 was run out with 5% propanol-cyclohexane. The remaining fractions were run out by development of the chromatogram with 30% propanol-cyclohexane. The qualitative appearance of the bands during the analysis of all four preparations was the same. Initial experiments indicated that fraction 1 required refractionation on a butanol-chloroform column to eliminate two components appearing as faint bands, running a little ahead of, and a little behind, the main acetylleucine band. Fractions 3 (alanine) and 4 (glycine) did not require further fractionation with butanol-chloroform.

to have a much lower M.P. (Found: C, 52·2; H, 7·97; N, 8·6%; acid equiv. wt. 163. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8%; acid equiv. wt. 159.)

(3) *Acetyl-l-alanine.* Crystallization from ethyl acetate-benzene yielded 12 mg. of a product having M.P. 125° (not depressed on admixture of acetyl-*l*-alanine derived from gelatin), $(\alpha)_D^{18°} - 37·2°$ (ethanol, $c = 0·4$). For the product from gelatin Gordon *et al.* [1943*b*] record M.P. 126–127°, $(\alpha)_D^{18°} - 36·9°$ (ethanol, $c = 0·4$). (Found: C, 46·4; H, 6·92; N, 10·6%; acid equiv. wt. 130. Calc. for $C_5H_9O_3N$: C, 45·8; H, 6·87; N, 10·7%; acid equiv. wt. 131.)

(4) *Acetylglycine.* Crystallization from ethyl acetate yielded 2 mg. of a product having M.P. 200° (not depressed on admixture of authentic acetylglycine, M.P. 205–206° [cf. Curtius, 1884]. The M.P. was depressed to 180° on admixture of acetyl-*l*-glutamic acid). There was barely sufficient material for an accurate determination of N and of acid equivalent weight, so no C, H determination was carried out. Together with the M.P. and chromatographic data, however, these analyses provide reasonable identification of the substance. (Found: N, 11·2%; acid equiv. wt. 123. Calc. for $C_4H_7O_3N$: N, 12·0%; acid equiv. wt. 117.)

Absence of serine from gramicidin

Serine was used in the known mixture employed as a control to the quantitative experiments described below. It always gave a distinct band during analysis, travelling at 0·55 time the rate of band 4 on the 30% propanol-cyclohexane column. This band was never observed during analyses of gramicidin. With the latter a much fainter band moving at 0·3–0·4 time the rate of band 4 was occasionally observed. Whether this was due to the hydroxy-amino compound of gramicidin further investigation will show.

Fraction X (Table 4) was regularly observed in the control experiments as well as in those with gramicidin; separate experiments showed it to be a product derived from tryptophan.

The two minor components of fraction 1, shown as occurring in gramicidin hydrolysates, were not observed during analysis of the known mixture.

Quantitative analysis of gramicidin hydrolysate

6–9 mg. of gramicidin were used for a single analysis, on chromatogram tubes of 1 cm. internal diameter. 3 g. of silica was used in each column.

After an estimate of the relative proportions of the various constituents had been formed, control analyses were carried out on a known mixture of acetamino-acids and serine, containing an amount of each constituent nearly equal to that present in the samples of gramicidin analysed, the same hydrolysis conditions and analytical procedure being used. Apart from the differences mentioned above, the chromatograms were indistinguishable from those obtained with gramicidin. The constituents of the mixture and the percentage recoveries are shown in Table 5. The acetyl derivatives were used in preference to the free amino-acids since, in addition to being of ascertained purity, their hydrolysis simulated more closely the hydrolysis undergone by gramicidin.

Table 5. *Control experiments on gramicidin analyses*

Component of mixture	Recovery (%)
Acetyl-*d*-leucine	101, 101
Acetyl-*l*-tryptophan	66, 73
Acetyl-*dl*-valine	104·5
Acetyl-*dl*-alanine	98, 101
Acetylglycine	78, 63
dl-Serine	—

Control experiments showed the high valine figure to be due to contamination by acetyltryptophan which 'tailed' during the separation of fractions 2·1 and 2·2 with 3% butanol-chloroform. This was caused by using, throughout these experiments, a batch of gel which had been aged for too long under acid [cf. Gordon *et al.* 1943*a*]. No such 'tailing' was noticed on the propanol-cyclohexane columns made with the same gel. Accordingly, in the analyses shown in Table 6, the valine figures have been calculated from the titrations for fraction 2·2 *less* 6·8% of the titration for fraction 2·1. No correction has been applied to the leucine and alanine figures.

The low tryptophan recoveries are due to destruction of tryptophan in the course of hydrolysis, etc.; the low glycine recoveries presumably to incomplete extraction of acetylglycine from the acidified acetylation mixture by the 250 ml. of 17% butanol-chloroform used. The figures for glycine and tryptophan in Table 6 have accordingly been corrected assuming these recoveries, and must be regarded as semi-quantitative only. Reserve is particularly necessary in assessing the tryptophan figures, since the destruction during hydrolysis of tryptophan residues in combination in gramicidin may be different from that occurring when they are present in acetyltryptophan.

Table 6 shows the analytical results for preparations B, C and D corrected in this way.

Table 6. *Amino-acid composition of gramicidin preparations*

(N as % of total N)

Preparation ...	B	C	D
Leucine	20·0	20·4	21·6
Tryptophan	40·1–44·8	40·1–44·8	33·5–37·2
Valine	17·0*	16·6	15·6
Alanine	9·8	10·4	11·5
Glycine	5·0–6·2	5·7–7·0	6·6–8·0

* Maximum figure, owing to accidental contamination of the material for analysis by acetylvaline. This figure has not been incorporated in the mean values given in Table 2.

In compiling Table 2, the mean values of the determinations on preparations B and C have been used.

SUMMARY

Gramicidin has been subjected to acid hydrolysis, and the presence in the hydrolysate of *d*-leucine, *l*-tryptophan, *dl*-valine, *l*-alanine and glycine has been established.

A quantitative determination of these components has been made, and from this, in conjunction with the data of other authors, it seems probable that the gramicidin molecule contains the following 24 residues: 6 leucine, 6 tryptophan, 5 valine, 3 alanine, 2 glycine and 2 of an unknown hydroxy-amino compound.

We are grateful to Dr R. J. Dubos and to Sir H. H. Dale, P.R.S., for making a supply of tyrothricin available to us, and to Dr B. C. J. G. Knight, whose suggestion led us to undertake this work. We wish to thank the Director and Council of the Wool Industries Research Association and the International Wool Secretariat for permission to publish this work.

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A Study of the Partial Acid Hydrolysis of Cow-hide Gelatin

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In a recent paper [Gordon, Martin & Synge, 1941] we described preliminary experiments aiming at the characterization of products of partial hydrolysis of proteins and through this at the elucidation of structural sequences in the intact protein molecule.

In the present paper we report further work in this direction, using partition chromatography [cf. Martin & Synge, 1941*b*; Gordon *et al.* 1943 *a*, *b*]. This procedure has made possible the semi-quantitative isolation of a number of peptides in the form of their acetyl derivatives. Peptide isolations have usually depended on the chance insolubility of a particular peptide or its derivative, and have given unknown yields. The new, more refined methods make possible a study of all the peptides of a given class occurring in a partial hydrolysate of a protein, and thus provide a more extensive picture than was formerly possible. The chromatographic procedure has been used simultaneously for rapid determination, on the micro scale, of the amino-acid composition of the peptide fractions isolated, an essential step too frequently neglected by workers in this field.

Apart from its value for the study of protein structure, this work may have applications in the field of serology, since Landsteiner [1942] has shown that the products of the partial hydrolysis of an antigenic protein can specifically inhibit its precipitin reactions with a homologous antiserum.

The present work was carried out with gelatin, since the absence of tryptophan, tyrosine and cystine from this material considerably simplifies the isola-

tive part of the investigation. This afforded us simultaneously a relatively simple check on current speculations as to the sequence of amino-acids in gelatin. The bearing of our experimental data on these speculations is discussed at the end of this paper.

Commercial gelatins are derived from the skin or bones of a number of species of animal, and are often mixtures in the biological sense. Because the parent collagens show significant differences in X-ray diffraction properties [cf. Astbury, 1940], it seemed particularly desirable to work with gelatin derived from a single source. The material used in the present work was a sample of cow-hide gelatin kindly placed at our disposal by Messrs Ross (Gelatines), Ltd., of Newlay, Leeds.

EXPERIMENTAL

(1) *Analytical methods*

Determinations of N (Kjeldahl), amino-N, free amino-acids (by ninhydrin) and tyrosine were carried out as previously [cf. Gordon *et al.* 1941]. The procedure used for determining monoamino-acids was as follows:

Determination of monoamino-acids. The chromatographic method previously described [Martin & Synge, 1941*b*] had, for the present work, been extended to deal with alanine, and in certain circumstances with glycine. It had further proved possible to differentiate valine and methionine from proline by determination of amino-N. The analysis was carried out as before [Gordon *et al.* 1943*a*] except that the series of refractionations with propanol-cyclohexane was omitted, the material from the butanol-chloroform column being titrated directly. 1% butanol-chloroform, followed by 17% butanol-chloroform after band 3 had run out, was used for development of the chromatograms. Phenolphthalein was used as indicator for the titrations.

The acetylalanine band was followed by that of acetylglycine. This was run out and titrated in the same way. As explained elsewhere [Gordon *et al.* 1943*a*], this titration represents glycine only if cystine, lysine, glutamic and aspartic acids are absent, otherwise, under these conditions, the acetyl derivatives of those acids move at about the same rate as acetylglycine. When the glycine band has moved some way down the column, any hydroxyproline present appears as a more slowly moving band. No attempt was made to run this out, but semi-quantitative estimates could be made on the basis of the colour and breadth of the band.

After titration with $Ba(OH)_2$ the proline-valine-methionine fraction was hydrolysed by refluxing for 5 hr. in 6*N* HCl at a volume of 10 ml. After this de-acetylation, excess HCl was removed *in vacuo*, and it was neutralized with Na acetate. Amino-N was then determined on the fraction. This made it possible to differentiate the proline from the valine and methionine. In the control experiment described below, where proline was separated from a mixture of amino-acids not containing valine or methionine, 0·232 mg. of proline-N in the fraction gave only 0·007 mg. amino-N. Similar experiments showed that under the same conditions valine and methionine gave 85–95% recovery of amino-N. This imperfect recovery, and the fact that in many experiments only very small amounts of valine and methionine were present, make it necessary to interpret the relevant figures with circumspection.

Influence of indicators. Methyl orange, phenolphthalein and baryta were always present in the final fractions prepared for amino-acid analysis (cf. § 6). As it was possible that these substances might give rise during hydrolysis to acid degradation products which might interfere with the chromatography, control experiments with regard to their decomposition were undertaken.

Methyl orange degradation products. 100 mg. of methyl orange were refluxed for 24 hr. in 6*N* HCl. Excess HCl was removed *in vacuo*. The resultant material was acetylated, and extracted with 17% butanol-chloroform as usual and one-fifth of this extract was taken for the chromatogram. This was run first with 1% and then with 17% butanol-chloroform as described above. During the first stage no acid band appeared. On development with 17% butanol-chloroform a faint band was seen to move down slightly behind the front of the new solvent. This was followed by a second very faint band which was estimated to move at a rate between that characteristic of glycine and that of hydroxyproline. Neither of these bands was strong enough to give a significant titration.

Phenolphthalein degradation products. 10 mg. of phenolphthalein were refluxed with acid, acetylated and extracted as above, and the whole of the extract put on the column. On development with 1% butanol-chloroform, a faint band moved down the column having $R=1{\cdot}0$. A second band was observed near the top of the column. On development with 17% butanol-chloroform this band moved down at $R=0{\cdot}15$. A third faint band moved at $R=0{\cdot}065$. Again none of these bands was of significant intensity.

Percentage recovery of amino-acids in a known mixture. Thus it appeared that indicators did not interfere with the analysis and a control experiment on amino-acid recovery was carried out. The amino-acids in the proportions shown in Table 1, together with 1 ml. of a saturated aqueous solution of methyl orange, 0·5 ml. of a 1% solution of phenolphthalein in ethanol and 0·33 ml. of *N*/3 $Ba(OH)_2$ were made up to 10 ml. in 6*N* HCl. The solution was refluxed for 36 hr. and after removal of excess HCl *in vacuo* the mixture was acetylated and extracted as described above.

One-tenth of the extract (corresponding to 1·10 mg. N in the original amino-acid mixture) was put on a column as above. The resultant analysis led to the recoveries shown in Table 1. The low

Table 1. *Percentage recovery in a known mixture of amino-acids*

Amino-acid	N as % of total N of mixture: Taken	Found	% recovery
dl-Phenylalanine	15·5	11·5	74
dl-Leucine	15·3	15·3	100
l-Proline	14·9	14·3	96
dl-Alanine	19·1	18·1	95
Glycine	16·6	13·6	82
l-Hydroxyproline	18·6	—	—
	100		

recovery of acetylglycine is explained by the incomplete extraction of this acid from the aqueous acetylation mixture. All figures given for glycine in the present paper have therefore been corrected assuming 82 % recovery.

The low recovery of phenylalanine was due to the use of gel containing traces of alkali. This question is dealt with by Gordon *et al.* [1943*a*]. The unsatisfactory gel used in the present experiment was also used in analysing the 'neutral peptide' fractions from the 4-day gelatin hydrolysate. Elsewhere in the present work gel giving satisfactory phenylalanine recoveries was used.

The above control experiment gives an indication of the accuracy of the analytical operations described in this paper.

(2) *Analysis of gelatin*

Monoamino-acids. Since the present investigation was concerned mainly with peptides of the monoamino-acids, an analysis for the total of each of these components was first carried out on a complete hydrolysate.

The gelatin was hydrolysed by refluxing for 24 hr. in 6*N* HCl. A colorimetric tyrosine determination on the hydrolysate showed some colour-producing substance to be present—this, however, was mainly associated with the small amount of insoluble humin in the hydrolysate—and no band corresponding to tyrosine was ever noticed on the chromatograms [cf. Gordon *et al.* 1943*a*].

The hydrolysate was analysed for constituent monoamino-acids. The results are shown in Table 2, together with our later figures for the same sample of gelatin [Gordon *et al.* 1943*a*] and those of the best previous determinations on gelatin. In converting these earlier figures to a N basis it was assumed that the N content of the gelatin (not recorded by Dakin or by Bergmann & Stein) was 18·3 % [cf. Beach & Teague, 1942].

Table 2. *Monoamino-acids of gelatin*

(N as % of total N)

Author ...	(1)	(2)	(3)	Others	
Phenylalanine	1·0	0·4	0·6	—	
Leucine isomers	3·9	3·9	4·1	—	
Valine	1·5	1·7	0	—	
Methionine		0·3	—	0·4	Beach & Teague [1942]
Proline	9·7	9·6	7·5	11·6	(4)
Alanine	9·0	8·3	7·5	—	
Glycine	—	—	26·0	27·0	(4)
Hydroxyproline	—	—	8·2	8·4	Bergmann [1935]

(1) Present determination.
(2) Gordon *et al.* [1943*a*].
(3) Dakin [1920].
(4) Bergmann & Stein [1939].

The difference between the phenylalanine value obtained here, and that obtained by our later procedure, shows clearly the necessity for the second fractionations with propanol-cyclohexane. In other cases our two analyses are in substantial agreement.

Methionine. Determinations on the present sample of gelatin, kindly carried out by Dr S. Blackburn of Torridon by the Baernstein volatile iodide procedure, gave a corresponding figure of 0·67 %. After the gelatin had been hydrolysed by 10*N* HCl at 37° for several weeks, this value had fallen to 0·14 %, suggesting demethylation. The possibility that methionine was oxidized to its sulphoxide by the H_2O_2 used in the manufacture of the gelatin should not be ignored.

Valine. The isolation, from the present sample of gelatin, of valine, in the form of its acetyl derivative, is described below (§ 6*d*). This is the first time valine has been shown by isolation to be a constituent of gelatin. Fischer, Levene & Aders [1902] obtained some evidence for its occurrence but left the question open pending more detailed investigation. Dakin [1920] asserted that it was absent. Dr R. Block (private communication) has obtained evidence for its occurrence in gelatin, using a modification of the procedure of Fromageot & Heitz [1939] [cf. Block & Bolling, 1941: we have not had access to this publication].

(3) *Course of hydrolysis*

The gelatin was hydrolysed in the same manner as before [Gordon *et al.* 1941], by allowing it to stand in 10*N* HCl at 37°, samples being removed at intervals for estimation of amino-N and free amino-acids. Fig. 1 shows a plot of these results. The large

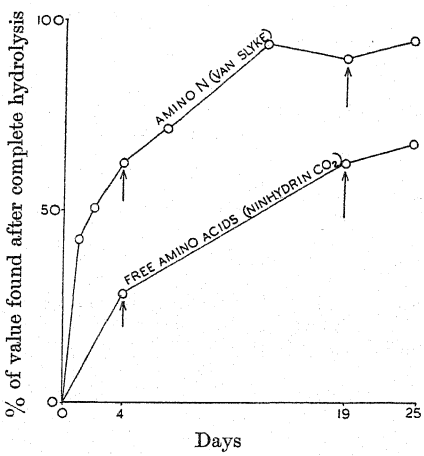


Fig. 1. Course of hydrolysis of gelatin in 10*N* HCl at 37°.

amount of glycine in gelatin makes it necessary to treat the amino-N figures with reserve. No correction for the glycine can be made because, although

the proper factor for the free amino-acid is well known, this is not the case when it is still in peptide linkage [cf. Van Slyke, 1911].

That the figure for free amino-acids had reached only a fraction of its final value when the figure for amino-N had almost reached 100% seems to indicate that the imino groups of proline and hydroxyproline are present, as previously suggested [Gordon *et al.* 1941], in peptide bonds resistant to acid hydrolysis at 37°.

The partial hydrolysates used for analysis were removed after 4 and 19 days. Further hydrolysis was stopped by removal of HCl by repeated evaporation with water *in vacuo*. The mixture was then diluted with water and stored at 0°. Fig. 1 shows that in the 4-day hydrolysate approximately one-third, and in the 19-day hydrolysate approximately two-thirds, of the residues had been converted to free amino-acids.

Table 3. *Analysis of fractions obtained by electrodialysis from partial hydrolysates of gelatin*

(N as % of total N of unfractionated gelatin)

	4 days' hydrolysis	19 days' hydrolysis
Unfractionated hydrolysate:		
Amino-N before complete hydrolysis	40·7	59·9
Amino-N after complete hydrolysis	67·0	67·0 (assumed)
Free amino-acids before complete hydrolysis	24·7	60·5
Free amino-acids after complete hydrolysis	87·0	87·0 (assumed)
Basic fraction:		
Total N	22·0	22·5
Amino-N before complete hydrolysis	6·3	7·6
Amino-N after complete hydrolysis	10·5	9·6
Free amino-acids before complete hydrolysis	2·3	5·5
Free amino-acids after complete hydrolysis	9·6	8·4
Neutral fraction:		
Total N	51·6	62·6
Amino-N before complete hydrolysis	25·3	37·8
Amino-N after complete hydrolysis	38·2	45·1
Free amino-acids before complete hydrolysis	16·2	45·7
Free amino-acids after complete hydrolysis	50·0	60·7

(4) *Preparation of 'basic' and 'neutral' fractions*

The electrodialysis of the partial hydrolysates was conducted in a diaphragm cell as previously described [Gordon *et al.* 1941], except that, in addition to the basic fraction, a neutral fraction was prepared. This consisted of the contents of the middle compartment of the cell after the first electrodialysis at *p*H 6. To ensure complete removal of basic and acidic material, the current was switched on again for a few minutes after the end compartments had been washed out, and refilled with distilled water. This was repeated a second time. Since some neutral material diffused out of the middle compartment during the preparation, the recovery of neutral amino-acids and peptides in this fraction was not quantitative. The amounts of the different substances lost depended on their different rates of diffusion, but only very slight changes in the relative amounts of the various molecular species left in the middle compartment, are to be expected.

In the case of the 'basic' fraction [cf. Gordon *et al.* 1941], the recovery should be quantitative.

The distribution of total N, amino-N, and free amino-acids in the partial hydrolysates and the fractions derived from these by electrodialysis is shown in Table 3. Amino-N and free amino-acids were determined before and after completely hydrolysing the material by refluxing for 24 hr. in 6*N* HCl. Comparison of these figures gives a measure of the amount of peptide material present in the different fractions.

The further analyses of the 'basic' and 'neutral' fractions are described separately below.

The above figures for free amino-acids are residues (1 mol. CO_2 evolved in presence of ninhydrin) expressed as N (1 atom) as % of the total N of the original unfractionated gelatin.

(5) *Investigation of the 'basic' fractions*

The 'basic' fractions obtained from the 4- and 19-day hydrolysates were analysed for monoamino-acids after complete hydrolysis, by the chromatographic method described above (§ 1). This experiment was similar to the analysis of the 'basic' fractions derived from various proteins [cf. Gordon *et al.* 1941], but owing to improved technique it could be carried out quantitatively. The amounts of the various amino-acids found are shown in Table 4. Assuming that these monoamino-acids are all linked as dipeptides with basic amino-acids, the yields from the 19-day hydrolysate account nearly quantitatively for the residues present in peptide linkage as determined by the ninhydrin procedure. Note also that the total amount of phenylalanine, leucine, valine and methionine residues bound in basic peptides after 4 days' hydrolysis is substan-

tially the same as after 19 days' hydrolysis. This is not, however, true for proline and alanine residues, and suggests that the bonds involving the former amino-acids are much more resistant to such hydrolysis than are those involving others such as proline and alanine.

Table 4. *Analysis of 'basic' fractions*

(N as % of total N of unfractionated gelatin)

Amino-acid	4 days' hydrolysis		19 days' hydrolysis	
Phenylalanine	0·05	0·56	0·04	0·59
Leucine	0·29		0·29	
Valine + methionine	0·22		0·26	
Proline	0·96	1·73	0·35	0·65
Alanine	0·77		0·30	
Total	2·29		1·24	
Residues in peptide linkage (moles as N by difference of free amino-acid determinations: cf. Table 3)	7·29		2·9	

(6) *Investigation of the 'neutral' fractions*

(*a*) *Chromatographic fractionation.* Preliminary experiments with synthetic acetyldipeptides [Gordon *et al.* 1943*a*] showed that these compounds are as readily separable by partition chromatography as are acetamino-acids, and that in general, for a given solvent pair, the acetylpeptides travel at rates intermediate between those for the acetyl derivatives of their constituent amino-acids.

In the present work on neutral peptides from gelatin, we have used successive fractionation on ethyl acetate-water and chloroform-butanol-water columns. This gives specially sharp separations of proline, whose acetyl derivative moves at the same rate as acetylvaline in chloroform-butanol, but moves more slowly than acetylalanine in ethyl-acetate. The proline peptides in partial hydrolysates of gelatin behave similarly, and are therefore specially amenable to study by the technique described here.

The 'neutral' fraction resulting from electrodialysis of the partial hydrolysate (corresponding to about 0·8 g. of gelatin) was evaporated to dryness *in vacuo* below 40°, acetylated, acidified and extracted with 17% butanol-chloroform as in the case of the monoamino-acid determination. The 4-day hydrolysate had 73 mg. N in the solution taken for the acetylation. In the 19-day hydrolysate 78·5 mg. N was present, 73% of which was obtained in the extract by this procedure.

The extracted material was fractionated on an ethyl acetate-water column of diameter 4·5 cm. Where the band was relatively fast-moving, and had 'run out' at the bottom of the column, the fraction was collected by evaporation of the solvent *in vacuo*. To obtain the slower moving fractions the column was pushed out of its tube after the developing solvent had run through, and each 'band' cut out immediately with a palette knife. Each fraction was immediately

Table 5. *Fractionation of 'neutral' material from 4-day hydrolysate*

	Fractionations			
	$R_{ethylacetate}$	$R_{butanol\text{-}chloroform}$ 1% butanol	$R_{butanol\text{-}chloroform}$ 17% butanol	Final fractions
Acetylated extract	>1	0·5	—	I
	0·9	0·3	—	II
		0·15	—	III
		0·1	—	Discarded
		0·07	—	Discarded
	0·52	0·5	—	Discarded
		0·15	—	IV
		0·05	—	V
		—	0·24	Discarded
	0·3*	—	1·2	VI
		—	0·4	VII
	0·15	—	0·7	VIII
		—	0·45	IX
		—	0·23	X
	<0·08*	—	0·6	XI
		—	0·4	XII
		—	0·13	XIII

* These fractions left a residue insoluble in hot 17% butanol-chloroform which was not further fractionated.

Table 6. *Fractionation of 'neutral' material from 19-day hydrolysate*

	Fractionations			
	$R_{ethylacetate}$	$R_{butanol\text{-}chloroform}$ 1% butanol	$R_{butanol\text{-}chloroform}$ 17% butanol	Final fractions
Acetylated extract	1·5	0·5	—	1
	1·35	0·5	—	2
		0·25	—	3
	0·9	0·15	—	Discarded
		0·10	—	4
		0·02	—	5
	0·6	0·15, 0·08	—	6
		—	0·4	Discarded
	0·26	—	0·7	7
		—	0·4	8†
	0·16	—	0·9	9
		—	0·3	10†
		—	0·1	11
	0·13	—	0·8	12
		—	0·15	13†
	0·08	—	0·8	Discarded
		—	0·5	14
		—	0·32	15
		—	0·19	16
	0·05	—	0·51	17
		—	0·42	18
		—	0·30	Discarded
		—	0·18	19
		—	0·13	20
		—	0·07	21
	0·04	—	0·4	22†
		—	0·07	23
	Slowest	—	0·46	24
		—	0·34	25
		—	0·17	26
		—	<0·12	Not analysed

eluted from the gel by thorough washing with alcohol on a Buchner funnel and the eluate evaporated to dryness *in vacuo*.

Every fraction so obtained was further fractionated on a chloroform-butanol-water column of suitable size and developed successively with solvent containing 1 and 17% of butanol v/v. The final fractions were collected in exactly the same way. For details see Tables 5 and 6.

In Tables 5 and 6 the final fractions, to each of which a number has been assigned, are tabulated. Several fractions were discarded, as too small. The symbol ⌒ between two fractions indicates grossly incomplete resolution of the corresponding bands; ⌒ indicates less serious mutual contamination, judged visually from the separation of the bands; while † indicates that the corresponding band was obviously due to more than one substance, being either very diffuse, or having the point of maximum concentration not near the leading edge of the band. Further fractionation on different chromatograms would have led to purer products, but was omitted because the present work was intended mainly as a preliminary survey.

The fractionation of the material from the 19-day hydrolysate was carried out in much greater detail than the fractionation of that from the 4-day hydrolysate, because during the initial fractionation on an ethylacetate column, the former resolved into a greater number of separate bands, between which 'cuts' could be made. Presumably the 19-day hydrolysate is a less complicated mixture. As seen in Table 5, the 4-day hydrolysate gave no resolution of bands having $R_{\text{ethylacetate}} < 0{\cdot}08$, the column above this level being uniformly pink. In the 19-day hydrolysate, a number of satisfactory 'cuts' could be made, and thus fractions 14–26 in the 19-day hydrolysate correspond to fractions XI–XIII in the 4-day hydrolysate. This in part explains the impossibility of interpreting stoichiometrically the amino-acid figures for fractions XI–XIII.

(*b*) *Amino-acid analysis of the final fractions.* Each of the final fractions was dissolved in ethanol, and a suitable aliquot was taken for amino-acid analysis. After evaporation of ethanol *in vacuo*, the material was dissolved in a little water and titrated with $N/100$ $Ba(OH)_2$, using phenolphthalein as indicator. This titration gave a measure of the number of equivalents of acetylpeptide and/or acetamino-acid present, and served as a check on the subsequent amino-acid analysis, thus:

Acetylglycylproline (1 equiv.) —hydrolysis and acetylation→ acetylglycine (1 equiv.) + acetylproline (1 equiv.)

Acetylglycylglycine (1 equiv.) → acetylglycine (2 equivs.)

HCl was then added to the titration mixture so that the final volume (10 ml.) contained $6N$ HCl. The mixture was then refluxed for 24 hr. to effect complete hydrolysis. Monoamino-acids were determined as described in § 1 (see Table 7).

In any given fraction, the total quantity of each amino-acid is expressed as the percentage of the total N in the 'neutral' fraction of the corresponding partial hydrolysate of gelatin. The acid equivalents in the fraction before hydrolysis are expressed in the same units. The symbol * attached to an analytical figure indicates that in the fraction concerned the amino-acid in question occurred as its acetyl derivative and not as a peptide, and hence could be assumed to be in the free form in at least this amount in the partial hydrolysate. This is deduced from the analytical figures for the fraction in question, and is confirmed by (*a*) chemical characterization (see § 6*d*) and (*b*) the fact that the rate of travel on the chromatogram agrees with that expected for the particular acetamino-acid [cf. Gordon *et al.* 1943*a*].

The analyses showed no bands other than those corresponding to the known acetamino-acids and 'indicator artefacts', suggesting that the treatment with acid resulted in complete hydrolysis of all peptide material. In a few cases the 'glycine' band appeared to be complex, suggesting that lysine, glutamic or aspartic acids might be present. Peptides of lysine with either glutamic or aspartic acids should occur in the 'neutral' fraction resulting from electrodialysis.

The symbols 'ftr' (faint trace), +, etc., in Table 7 are relative to the total amount of material in the fraction submitted to analysis, and not with respect to the original gelatin.

Apart from fractions marked with an asterisk, in which single acetamino-acids predominate, the analyses in Table 7 suggest that the final fractions are still, in the majority of cases, complicated mixtures of acetylpeptides. Speculation as to the peptides present must be based, first, on the correspondence between the rates of travel of the band corresponding to the fraction on the different chromatograms and those of known acetylpeptides and acetamino-acids, and secondly on stoichiometry. Serine and threonine, for which the fractions have not been analysed, may be present in some of them, and may make interpretation yet more difficult.

The following fractions seem the only ones of reasonably obvious composition:

4-day hydrolysate

Fraction V. Mainly dipeptide glycine-leucine. The corresponding fraction 6 from the 19-day hydrolysate probably contains the same substance though more heavily contaminated.

Table 7. *Amino-acid analysis of final fractions*

Fraction no.	Acid equivs. before hydrolysis (as N)	N as % of N of 'neutral' fraction of corresponding gelatin hydrolysate: Phenyl-alanine + leucine + valine + methionine + proline	Phenyl-alanine	Leucine	Valine + methionine	Proline	Alanine	Glycine	Hydroxy-proline
				4-day hydrolysate					
I	0·09	0·09	0·09*	0	0	0	0	0	0
II	0·40	0·40	0	0·40*	0	0	0	0	0
III	0·77	0·70	0	ftr	0·63*	0·07	0·04	0·04	0
IV	0·25	0·28	0·14	0·12	0·02	→	0·05	0·16	0
V	0·79	0·66	0·08	0·58	ftr	→	0·08	0·59	+
VI	3·54	3·76	0·86	0	0·78	2·12*	0·28	0·50	+ +
VII	4·90	0·87	0	0·29	0·58	→	3·85*	0·82	0
VIII	0·36	0·41	0	0·18	0·05	0·18	tr	0·13	0
IX	0·23	0·23	0	0·08	0·04	0·11	0·10	0·07	0
X	11·7	—	fftr	ftr	fftr	→	0·61	11·0*	0
XI	0·60	0·78	0	ftr	0·12	0·66	0·23	0·23	+ +
XII	4·10	4·20	0·70	0	1·05	2·45	1·45	3·38	+ +
XIII	5·37	2·02	0	0·29	0·25	1·48	1·94	2·34	+ + + + *
				19-day hydrolysate					
1	0·29	0·24	0·24*	tr	ftr	→	ftr	tr	ftr
2	0·47	0·41	0·41*	fftr	tr	→	ftr	tr	ftr
3	1·99	1·7	ftr	1·7*	fftr	→	fftr	ftr	fftr
4†	0·46	0·37	fftr	tr	0·36*	0·01	tr	tr	tr
5	0·32	0·18	0·06	0·07	0·05	ftr	0·06	0·11	0
6	1·13	1·06	0·17	0·67	0·14	0·08	0·21	0·61	+ +
7	0·42	0·42	0·04	0·20	0·17	0·01	0·12	0·11	+ +
8	10·4	—	0	fftr	tr	→	9·0*	1·15	fftr
9	8·15	6·79	0	fftr	0·39	6·4*	0·52	tr	+ +
10	0·56	0·30	0	0	0·20	0·10	0·17	0·21	+ +
11	0·64	0	0	0	0	0	0·05	0·64*	+
12	1·29	1·14	0	0	0·06	1·08*	ftr	tr	tr
13	23·3	—	0	0	fftr	→	tr	23·6*	0
14	0·19	0·12	0·02	0·03	0·04	0·03	0·02	0·04	+ + +
15	0·21	0·03	0	ftr	0·03	→	0·05	Lost	+ +
16	0·60	—	ftr	fftr	ftr	→	0·04	0·20	+ + +
17	0·06	0·06	0	tr	0·01	0·05	0·04	0·05	+
18	0·28	0·28	fftr	ftr	0·03	0·25	0·26	0·03	+
19	0·23	—	0	ftr	tr	→	0·07	0·06	+ + +
20	0·90	0	0	0	0	0	0·11	0·70	+ + +
21	1·17	—	0	0	fftr	→	0·06	ftr	+ + + *
22	1·20	1·05	0	0	0·09	0·96	0·22	0·81	+
23	2·76	—	0	fftr	tr	→	ftr	tr	+ + + *
24	0·14	0·18	0	0	0	0·18	tr	0·02	+ + +
25	0·27	0·22	ftr	0	0·03	0·19	0	tr	+ +
26	0·53	0·51	0	0	0·08	0·43	0·03	0·14	+ + +

† Minimum figures, as loss of unknown amount occurred in working up this fraction.

19-day hydrolysate

Fraction 17. Mainly tripeptide proline-alanine-glycine.

Fraction 18. Mainly dipeptide proline-alanine.

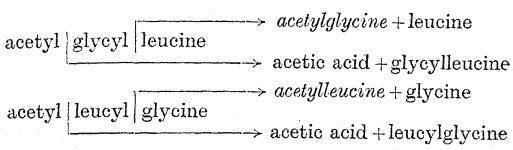
Fraction 22. A mixture of dipeptides proline-alanine and proline-glycine, with the latter predominating. The resolution of these substances seemed to have been achieved on the original ethyl-acetate-water chromatogram, with the result that fraction 18 was free from proline-glycine dipeptide, so some of fraction 22 was further fractionated on an ethylacetate-water column. Two bands were obtained. By cutting between these, two fractions were obtained—22*a* ($R_{\text{ethylacetate}} = 0{\cdot}063$) and 22*b* ($R_{\text{ethylacetate}} = 0{\cdot}050$). The band corresponding to 22*b* was much the stronger. Resolution of the bands did not appear to be perfect, and on amino-acid analysis of 22*b* it was found that 10 % of alanine-proline dipeptide was still present with the glycine-proline dipeptide.

Fraction 24. Perhaps dipeptide proline-hydroxy-proline.

(c) *Determination of order of amino-acid residues in peptides.* Recrystallization and formal identification of the various acetyl peptides described above was impracticable owing to the small amounts at our disposal. The fractionations shown in Tables 5 and 6 were, for the sake of rapid working, each carried out on hydrolysates of less than 1 g. of gelatin.

We have, however, obtained some evidence as to the order of arrangement of the amino-acid residues in some of the peptides, a point on which the analyses given above yield no information. The following is a preliminary description of the chromatographic micro-method which we are developing for this purpose.

When an acetyldipeptide is subjected to partial hydrolysis, the initial splitting can occur in one of two ways, of which one leads to the formation of an acetamino-acid:



Thus acetylglycylleucine on partial hydrolysis should yield acetylglycine but no acetylleucine, and acetylleucylglycine *vice versa*. These acetamino-acids may be separated from the hydrolysis mixture by extraction and chromatography, and quantitatively determined.

Experiments were accordingly undertaken on synthetic specimens of these two acetyldipeptides [Gordon *et al.* 1943*a*], and in the light of the results of these, some of the fractions derived from gelatin were investigated.

(1) Acetyl-*dl*-leucylglycine was hydrolysed for 36 hr. in 10*N* HCl at 37°. An aliquot of this partial hydrolysate, corresponding to 7 mg. of acetyldipeptide was evaporated to dryness *in vacuo* below 40°, and stored overnight in a vacuum desiccator over H_2SO_4 and soda-lime to effect complete removal of acetic acid and excess HCl. The product was then analysed on a chromatogram, giving:

Acetylleucine	0·14 mol. per mol. of starting material
Acetylleucylglycine	0·13 mol. per mol. of starting material
And no acetylglycine	

(2) A similar experiment with acetylglycyl-*dl*-leucine gave:

No acetylleucine	
Acetylglycylleucine	0·40 mol. per mol. of starting material
Acetylglycine	0·26 mol. per mol. of starting material

(3) On the basis of these experiments a portion of fraction V (glycine-leucine dipeptide) was subjected to the same procedure, and gave:

Acetylleucine	0·10 mol. per mol. of starting material
Starting material	0·22 mol. per mol. of starting material
Acetylglycine	0·15 mol. per mol. of starting material

This suggests that the peptides glycylleucine and leucylglycine are simultaneously present in the partial hydrolysate.

(4) A similar experiment was carried out with fraction 22*b*. This gave:

No acetylproline	
Starting material	0·14 mol. per mol. of starting material
Acetylglycine	A faint trace

Exp. 4 suggests that we are dealing with acetylglycylproline, but is inconclusive owing to the small amount of acetylglycine formed. Exp. 2 shows that acetylglycine is comparatively resistant to hydrolysis under these conditions—and could therefore not have been formed in significant amount at any stage. The most plausible explanation is that the hydrolysis proceeds almost entirely at the bond between the acetic acid and glycine residues, thus rendering the method inapplicable. This result accords with the view of enhanced stability to acid hydrolysis of peptide bonds involving proline imino groups which we have postulated above (§ 3) to explain the course of hydrolysis of gelatin itself under the same conditions. We have not yet conducted experiments using synthetic acetylglycylproline to check this hypothesis.

The method described in outline above may prove of value for the location of free amino groups in more complicated peptides and in proteins. Extraction from acid aqueous solution by suitable organic solvents of the partial hydrolysis products of acetylated proteins or peptides should yield a mixture of acetylated amino-acids and peptides. Amino-acid analysis of a complete hydrolysate of this extract should give information as to the terminal amino-acids of the peptide chains and in the case of a substance consisting of a single unbranched acetylated peptide chain, e.g. $Ac.x_1.x_2.x_3...$ should give the order of the residues directly, the quantities found being in the order $x_1 > x_2 > x_3$, etc.

The hydrolytic agent need not be an acid, but could be alkali, or, if rearrangement is out of the question, an enzyme. Other acyl groups than acetyl may also prove of value for the method.

(*d*) *Chemical identification of monoamino-acids as acetyl derivatives.* The amounts of acetamino-acids, as opposed to acetylpeptides, resulting from the fractionations described above, were adequate for complete chemical characterization. All were obtained as the *l*(+) optical stereoisomer.

All M.P.'s are given uncorrected. N determinations were by the Kjeldahl procedure. C, H determinations were done for us by Dr G. Weiler, Oxford.

Acetyl-l-phenylalanine. Crystallization from $CHCl_3$ of a fraction obtained from 10 g. of gelatin in the same way as fraction I yielded 22 mg. having M.P. 167–168° (not depressed on admixture of authentic specimen), $(\alpha)_D^{18°} + 45{\cdot}6°$ (ethanol, $c = 0{\cdot}5$). Jackson & Cahill [1938] record M.P. 170–171°, $(\alpha)_D^{25°} + 47{\cdot}6°$ (ethanol, $c = 1$). (Found: C, 63·4; H, 6·08; N, 6·84%; acid equiv. wt. 208. Calc. for $C_{11}H_{13}O_3N$: C, 63·7; H, 6·28; N, 6·77%; acid equiv. wt. 207.)

This isolation of phenylalanine in the optically active state by an acetylation and extraction procedure provides a check on the isolation of the racemic material from a wool hydrolysate [Martin & Synge, 1941*a*] confirming that racemization did not

take place in the course of isolation. Phenylalanine in wool must therefore exist in the racemic state, or be very readily racemized during hydrolysis by boiling acid.

Acetyl-l-leucine. Crystallization from $CHCl_3$ of a fraction obtained from 10 g. of gelatin in the same way as II yielded 40 mg. having M.P. 180° (not depressed on admixture of authentic specimen), $(\alpha)_D^{15.5°} - 20.0°$ (ethanol, $c = 1.3$). Martin & Synge [1941*a*] record M.P. 183–184°, $(\alpha)_D^{24°} - 22.6°$ (ethanol, $c = 3.3$). (Found: C, 56.2; H, 8.37; N, 8.0%; acid equiv. wt. 180. Calc. for $C_8H_{15}O_3N$: C, 55.5; H, 8.67; N, 8.1%; acid equiv. wt. 173.)

If *iso*leucine were also present in gelatin, acetyl-*iso*leucine should be found in the mother liquors from the above crystallization, which we have not further investigated. *Iso*leucine was stated by Dakin [1920] to be absent from gelatin.

Acetyl-l-valine. Crystallization from ethylacetate-benzene of a fraction obtained from 10 g. of gelatin in the same manner as III yielded 13 mg. having M.P. 155–156·5° (not depressed on admixture of authentic acetyl-*l*-valine), $(\alpha)_D^{20°} + 2°$ (ethanol, $c = 0.4$). Gordon *et al.* [1943*a*] record M.P. 164°, $(\alpha)_D^{19°} + 4.0°$ (ethanol, $c = 2$). The present material was evidently racemized to a considerable extent. (Found: C, 52·7; H, 8·00%; acid equiv. wt. 165. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17%; acid equiv. wt. 159.) Our original N determination was lost and material did not suffice for accurate redetermination.

Acetyl-l-proline (*monohydrate*). Crystallization from wet ethylacetate of material from fractions 9 and 12 yielded 50 mg. having M.P. 80° (not depressed on admixture of authentic specimen), $(\alpha)_D^{19.5°} - 113.4°$ (water, $c = 1.3$, – rotation calc. for anhydrous compound). Martin & Synge [1941*a*] record M.P. 78°, $(\alpha)_D^{22°} - 114.0°$ (water, $c = 4.2$), and give references to previous determinations. (Found: C, 53·7; H, 7·07% (on anhydrous material.) Calc. for $C_7H_{11}O_3N$: C, 53·5; H, 7·01%.) (Found: N, 8·1; water of crystallization, 10·8%; acid equiv. wt. 170 (on hydrated material). Calc. for $C_7H_{11}O_3N.H_2O$: N, 8·0; water of crystallization, 10·3%; acid equiv. wt. 175.)

Acetyl-l-alanine. Crystallization from ethylacetate-benzene and ethylacetate successively of material from fraction 8 yielded 40 mg. having M.P. 126–127°, $(\alpha)_D^{19°} - 65.0°$ (water, $c = 0.7$), $[\alpha]_D^{18°} - 36.9°$ (ethanol, $c = 0.4$). Karrer, Escher & Widmer [1926] record M.P. 116°, $(\alpha)_D^{16°} - 45.6°$ (water, $c = 5$). Their material must have been grossly racemized as would be expected from their mode of acetylation. The acetyl-*l*-leucine described in the same paper was prepared in the same way, and was also largely racemic. (Found: C, 45·8; H, 7·00; N, 10·4%; acid equiv. wt. 136. Calc. for $C_5H_9O_3N$: C, 45·8; H, 6·87; N, 10·7%; acid equiv. wt. 131.)

Acetylglycine. Crystallization from ethanol of material from fractions 11 and 13 yielded 78 mg. having M.P. 202–203° (not depressed on admixture of authentic specimen). Curtius [1884] records M.P. 206°. (Found: C, 41·3; H, 6·01; N, 12·1%; acid equiv. wt. 120. Calc. for $C_4H_7O_3N$: C, 41·0; H, 5·98; N, 12·0%; acid equiv. wt. 117.)

(*e*) *Liberation of free amino-acids during the partial hydrolysis of gelatin.* From the figures marked with asterisks in Table 7 it is possible to deduce minimum figures for the amounts of free neutral amino-acids present in the hydrolysate after 4 and 19 days. Although losses of unknown extent occurred in the preparation by electrodialysis, of the 'neutral' fractions, they were probably similar for both the 4- and 19-day hydrolysates, and the *relative* amounts of the different amino-acids present were probably little affected in the course of isolation.

The figures are shown in Table 8, expressed as percentages of the number of residues of the same species present in the original gelatin. We base these latter figures on our present determinations shown in Table 2, and employ in addition the mean figure for glycine given in that table.

Table 8. *Free amino-acids in partial hydrolysates of gelatin*

(As % of total residues of same species present)

Amino-acid	4-day hydrolysate	19-day hydrolysate
Basic amino-acids*	32	78
Phenylalanine	5	40
Leucine	5	27
Valine + methionine	21	†
Proline	11	48
Alanine	23	63
Glycine	21	57

* The free basic amino-acids have been calculated from the data in Table 3, and are expressed as a percentage of the total arginine + histidine + lysine residues determined by Dakin [1920], assuming for gelatin an N content of 18·3%. No loss is associated with these figures for basic amino-acids, since the 'basic' fraction is obtained quantitatively by electrodialysis (cf. § 4).

† Not calculated owing to loss involved in isolation of fraction 4 (cf. Table 7).

It will be seen that the different amino-acids are liberated at significantly different rates as the hydrolysis proceeds.

DISCUSSION

Earlier work on the isolation of the products of partial hydrolysis of gelatin and other proteins will shortly be reviewed by one of us (R. L. M. S.). Here it will suffice to mention the isolation by previous workers of diketopiperazines corresponding to some of the dipeptides isolated in the present study. These former resulted either from enzymic hydrolysis or from treatment with hot dilute acids. Our hydrolysis with cold concentrated acid did not give rise to such products, which, had they been present, would have been isolated, since they are extractable by organic solvents [cf. Gawrilow & Lawrowsky, 1927], and would have raised the amino-acid content of the final fractions far above the acid equivalent determined by titration. We deduce that

these diketopiperazines are artefacts arising by condensation from the corresponding dipeptides.

Glycine-proline anhydride was isolated from enzymic digests by Levene [1910] and from acid hydrolysates by Gawrilow & Lawrowsky [1927] and Konuma [1938]. Gawrilow & Lawrowsky also obtained evidence for the occurrence of glycine-leucine anhydride. Dakin's [1920] proline-hydroxyproline anhydride more probably arose from condensation of free amino-acids in butanol than from a corresponding dipeptide, although the present work gives indications of a dipeptide of proline and hydroxyproline (fraction 24).

The results we have obtained are not inconsistent with the structure suggested by Astbury [1940] (cf. Bergmann [1935]) for gelatin. One-eighteenth of the total residues have not been allotted a definite place in this structure, and such peptides as we have found (leucylglycine *and* glycylleucine and peptides of bases with neutral amino-acids) which could not be fitted into the main arrangement do not total one-eighteenth of the whole. To reconcile our data with Astbury's structure for gelatin it is necessary to make the assumption that the acid hydrolysis of bonds involving these 'anomalous' residues proceeds more slowly than for the others, and we believe that the structure will be found to be much more complicated than Astbury suggests.

Apart from the arrangement of the small number of peptides for which we have obtained definite evidence, we have noted what may be an underlying structural principle concerning the arrangement of the amino-acid residues in the peptide chain; i.e. monoamino-acids with longer fatty side-chains (phenylalanine, leucine, proline, valine and methionine) apparently are not linked to one another in gelatin. This seems to be established: (1) by the almost complete absence of acetylpeptides giving bands running faster than acetylvaline on the chromatogram. From the behaviour of synthetic acetylleucylleucine [cf. Gordon *et al.* 1943*a*] we expect that bands due to this type of peptide will move faster than that of acetylvaline; (2) by the fact that in no fraction does the sum of the phenylalanine + leucine + proline + valine + methionine equivalents (cf. Table 7) exceed the number of acid equivalents before hydrolysis, as would be the case if two residues from this group of acids were in the same molecule.

Being a negative principle, it is less easy to establish, and we are tentative in drawing conclusions. But it seems that this spacing out of fatty residues along the peptide chain may determine some of the peculiar physical properties of gelatin, and further that the principle of distribution of amino-acids by physical classes [cf. Astbury, 1942] may be of general significance with regard to protein structure.

SUMMARY

1. An investigation has been made of the partial hydrolysis of cow-hide gelatin by 10*N* HCl at 37°.

2. 'Basic' and 'neutral' fractions have been isolated from the resulting mixtures by electrodialysis at *p*H 6.

3. Analysis of the 'basic' fractions strongly suggests that residues of basic amino-acids occur in gelatin directly linked to residues of the higher monoamino-acids.

4. Analysis of the 'neutral' material by acetylation followed by fractionation on silica gel chromatograms has led to the isolation of a number of lower peptides in the form of their acetyl derivatives.

5. By similar means the gelatin has been analysed quantitatively for its constituent monoamino-acids, and these have been formally identified in the form of their acetyl derivatives. *l*-Valine has been shown to be present, and the rates of liberation of the various amino-acids during the partial hydrolysis have been studied.

6. A chromatographic micro-method has been developed for the determination of the order of amino-acid residues in acetylpeptides, etc.

7. The relation of the present study to previous work on gelatin, and to current theories of gelatin structure is discussed. It is suggested that in gelatin the higher monoamino-acids do not occur in juxtaposition along the peptide chain.

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Fatty Acid Dehydrogenase in Adipose Tissue

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It has been established in recent years that adipose tissue in the body is not an inert site of fat deposition but a tissue with specific metabolic functions. This is proved by its respiratory activity [Mirski, 1942] and by its ability to deposit glycogen under suitable conditions [Hoffmann & Wertheimer, 1927; Tuerkischer & Wertheimer, 1942]. Further proof is given by experiments showing the dependence of various adipose functions upon innervation [Hausberger, 1937].

It might be assumed, in view of these results, that adipose tissue plays an important part in the deposition and mobilization of fat. Investigation of the fat metabolism of this tissue may therefore well prove of interest for the understanding of the deposition and mobilization processes.

The present investigation arises from the observation that aqueous extracts of adipose tissue decolorize methylene blue, the speed of the decolorization being enhanced when fatty acids are added. It was proposed, therefore, to prepare an active fatty acid dehydrogenase from adipose tissue and to study the properties of the isolated enzyme.

Towards the conclusion of this work, abstracts of papers by Lang [1939], Lang & Mayer [1939] and Lang & Adickes [1939], appeared in which the demonstration of fatty acid dehydrogenase in various sources is reported. As the original papers are not at present available to us, no comparison of methods is possible. The authors report that the dehydrogenase action is dependent on the availability of muscle-adenylic acid as coenzyme and that the activity is greater to high than to low fatty acids.

METHODS

Preparation of enzyme solution. Adipose tissue from the leg region and the abdominal cavity of rats is cut into small pieces with scissors and crushed with twice its weight of sand. The pulp thus obtained is extracted with 2 volumes of distilled water for $\frac{1}{2}$ hr. at room temperature and centrifuged. The supernatant is freed of fat by filtration. The liquid is then dialysed through cuprophan for 6–20 hr. against running tap water. Adipose tissue of rabbits did not yield active extracts.

Boiled tissue juice was prepared by boiling 2 g. of adipose tissue pulp with 5 ml. water in a water bath for 30 min. The juice is filtered while hot.

Crude cozymase preparation was prepared according to Euler [1931]. Solutions obtained after cleavage of the lead precipitate with H_2S were used for the experiment.

Dehydrogenase activity estimations were carried out according to Thunberg. Test mixtures consisted of 0·1 ml. $M/2$ phosphate buffer pH 8·0, 0·1 ml. methylene blue (1/5000), with the following additions: 0·5 ml. enzyme solution, 0·1 ml. substrate ($M/20$) and 0·1 ml. coenzyme. When one of these constituents was not added it was replaced by distilled water. Fatty acids in the experimental mixture were neutralized with NaOH and brought to pH 8·0. In some cases, dehydrocholic acid was used for dispersion, as it had no influence on the dehydrogenase activity.

EXPERIMENTAL

As is shown in Table 1, extracts dialysed for 20 hr. did not cause decolorization of methylene blue even after the addition of fatty acids. However, when boiled tissue juice was added decolorization took place. The speed of decolorization was markedly enhanced by addition of fatty acids. Crude cozymase instead of tissue juice may be used with even better results. Neither tissue juice nor cozymase preparation caused decolorization when added to fatty acids but without enzyme. When enzyme solution boiled for 5 min. was used decolorization failed to take place even in the presence of coenzyme and substrate. The enzyme nature of the dehydrogenase is thus established.

Table 1. *Enzymic reduction of methylene blue*

Addition to enzyme soln.	Time of decol. min.
None	No decol.
Stearate	,,
Boiled juice	70
Boiled juice + stearate	30
Crude cozymase	90
Crude cozymase + stearate	12
Boiled enzyme + crude cozymase + stearate	No decol.
Boiled juice + stearate	,,
Crude cozymase + stearate	,,

In view of the report of Lang & Mayer [1939], experiments were undertaken to examine whether muscle adenylic acid can replace boiled tissue juice or crude cozymase as coenzyme. This was found to be the case. Inosinic acid can also act as coenzyme. Yeast adenylic acid did not display coenzyme activity.

Table 2. *Influence of coenzyme on reduction of methylene blue*

Addition to enzyme soln.	Time of decol. min.
None	No decol.
Palmitate	,,
Muscle adenylic acid (20 mg. %)	120
Muscle adenylic acid (20 mg. %) + palmitate	3
Inosinic acid (20 mg. %)	36
Inosinic acid (20 mg. %) + palmitate	3
Yeast adenylic acid + palmitate	No decol.

As is shown in Table 2, although inosinic acid can act as coenzyme, the use of adenylic acid is preferable, as inosinic acid itself caused comparatively rapid decolorization even in absence of substrate. Diphosphopyridinenucleotide does not seem to be involved, since adipose tissue extracts capable of serving as coenzyme of fatty acid dehydrogenase could not activate lactic dehydrogenase of muscle. In addition to coenzyme, inorganic phosphate must be present for this dehydrogenase action. When the phosphate buffer is replaced by bicarbonate, dehydrogenation is comparatively slow, as is shown in Table 3.

Table 3. *Effect of phosphate on dehydrogenation*

Addition to enzyme soln.	Time of decol. min.
Adenylic acid + phosphate	90
Adenylic acid + phosphate + palmitic acid	4
Adenylic acid + 1·3 % bicarbonate	110
Adenylic acid + 1·3 % bicarbonate + palmitic acid	75

p*H optimum of fatty acid dehydrogenase*

In determining the optimum *p*H for the dehydrogenase, difficulty was encountered in keeping the fatty acid in suspension below *p*H 8. This was overcome by the use of Na dehydrocholate as dispersing agent. Stearic acid was dispersed in 0·5 % Na dehydrocholate and the reaction brought to the required *p*H with NaOH. By this means a comparatively stable suspension could be obtained at

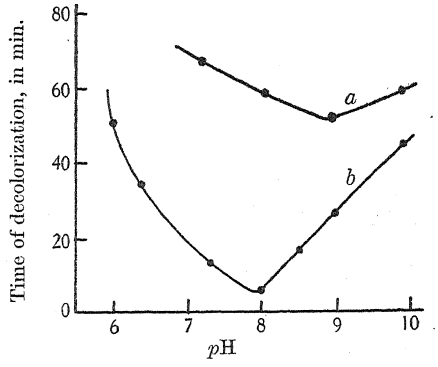

Fig. 1. *a*, without substrate. *b*, with stearic acid.

as low a *p*H as 6·0. Controls containing the same amount of Na dehydrocholate were done. The results are shown in Fig. 1, which demonstrates a clear optimum of dehydrogenase action at about *p*H 8.

Substrate specificity

The activity of the enzyme towards fatty acids of different chain lengths, with even and uneven number of carbon atoms, as well as towards other substrates, was examined. A crude cozymase preparation was used in these experiments as coenzyme. The results are summarized in the following table.

Table 4. *Specificity of dehydrogenase*

Addition	Time of decol. min.
Enzyme + no substrate	90
+ stearate	2
+ palmitate	3
+ caprinate	10
+ capronate	10
+ valerate	25
+ butyrate	33
+ oleate	15
+ succinate	5
+ lactate	30
+ glycerophosphate	18
+ lecithin (sheep brain)	10
+ cephalin (sheep brain)	12
No enzyme + lecithin	>180
+ cephalin	>180
+ oleate	>180

The table shows clearly that with diminishing fatty acid chain length the enzyme activity decreases. The unsaturated fatty acid oleic acid is dehydrogenated much more slowly than the corresponding saturated acids. Also phospholipins are

dehydrogenated by the enzyme. Separate experiments confirmed that with phospholipins, as with fatty acids, the same coenzyme and inorganic phosphate are needed for dehydrogenase action. The optimal *p*H for fatty acid was found to be the same as for phospholipins. Of other substrates tested, succinate alone was found to be dehydrogenated at a rate comparable with that of the fatty acids.

In order to ascertain whether the fatty acid dehydrogenase was able to act on neutral fat, a dispersing agent other than fatty acid had to be employed. A suspension of neutral fat in 1 % lauryl alcohol sulphonate was used. As Table 5 shows this agent had no inhibiting effect on fatty acid dehydrogenation. Nevertheless, no decolorization of methylene blue was observed with a suspension of neutral fat.

Table 5. *Action of dehydrogenase on neutral fat*

Addition to enzyme soln.	Time of decol. min.
No substrate + coenzyme	75
Palmitate + coenzyme	4
Palmitate + coenzyme + lauryl alcohol sulphonate	4
Neutral fat in lauryl alcohol sulphonate + coenzyme	>120

Inhibitors of dehydrogenase action

In view of the fact that inorganic phosphate was necessary for dehydrogenase action, a number of poisons of phosphorylating reactions were examined as to their effect on fatty acid dehydrogenation. Other dehydrogenase poisons were also tested. In no case could an inhibiting action of these substances be observed. The substances and concentrations tested were: monoiodoacetate *M*/100, phloridzin *M*/100, NaF *M*/50, maleic acid *M*/200, pyrophosphate *M*/200 and benzoate *M*/100.

These results, as well as the impossibility of demonstrating any decrease in inorganic phosphate during dehydrogenase action, seem to exclude the possibility that the activating action of phosphate on the dehydrogenase involves phosphorylation.

The finding that maleate and pyrophosphate failed to inhibit decolorization is evidence against the identity of fatty acid dehydrogenase with succinic dehydrogenase.

Distribution of enzyme

An enzyme which desaturates fatty acids could be demonstrated in liver, muscle, heart, testes, brown fat as well as in adipose tissue. No activity could be demonstrated in brain. With these tissues, phospholipins were dehydrogenated at the same speed as fatty acids, in contrast to adipose tissue. Activity in these tissues was not as clearly demonstrated as in adipose tissue. This was due to the presence of other active dehydrogenases with their substrates, which caused decolorization in the absence of fatty acids. In order to remove all hydrogen donators prolonged dialysis (at least 24 hr.) was necessary. This treatment, however, also weakened the fatty acid dehydrogenase. Better results were obtained when the cut organs were washed with 0·9 % NaCl and then extracted with distilled water, followed by dialysis for 4–6 hr.

Influence of oxygen on dehydrogenase

Attempts to demonstrate dehydrogenase activity by measuring O_2 consumption in the presence of methylene blue were unsuccessful. This finding is explained by the fact that the enzyme mixed with coenzyme and substrate is inactivated in the presence of O_2. If, after mixing all components of reaction, the mixture is allowed to stand at room temperature in the air for 10 min. before evacuating, no activity can be observed even with Thunberg's method.

SUMMARY

1. A fatty acid dehydrogenase has been demonstrated in extracts of adipose tissue. Its activity depends on the presence of a coenzyme, adenylic acid, and inorganic phosphate.

2. The optimum *p*H of the dehydrogenase is 8·0.

3. Activity is highest with the natural, long chain, fatty acids and decreases with diminishing chain length.

4. The dehydrogenase also oxidizes phospholipins. Of other substrates tested only succinate was dehydrogenated at a rate comparable to palmitic and stearic acid. Neutral fat does not seem to be attacked.

5. Of tissues other than adipose tissue, liver, muscle, heart and testes, were proved to contain fatty acid and phospholipin dehydrogenase.

6. Monoiodoacetate, phloridzin, NaF, maleic acid, pyrophosphate and benzoate at fairly high concentrations do not affect the enzyme.

7. Exposure of the reaction mixture to O_2 caused rapid inhibition of the enzyme activity.

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Differences in Calcification between the Humerus and Femur of Young Rats receiving High Calcium, Low Calcium and Stock Diets

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The calcification of bones in both normal and pathological conditions, especially in rickets, is frequently described in terms of ash content in preference to histological structure. The methods used in the determination of bone ash vary little from one laboratory to another as regards the procedures for the extraction of the fat and the ashing of the bones, but the choice of the particular bone or bones to be examined is a matter of individual preference. Chick & Roscoe [1926] ashed femur, tibia and fibula together in the determination of the ratio mineral ash/organic residue as a rachitogenic index. Kon & Henry [1935], in carrying out prophylactic estimations of vitamin D, determined the percentage ash from one femur and one humerus together for each rat. Other workers have chosen one bone only as sufficiently representative, e.g. the tibia [Schultzer, 1931] or the femur [Bethke, Kick & Wilder, 1923]. In any of these procedures the final value for each rat is obtained from a single determination or from duplicate determinations made on right and left bones. Bethke *et al.* [1923] ashed separately the humeri and femora of each rat and obtained an average value from separate analyses of bones from different parts of the body.

In this laboratory it has been customary to follow Bethke's technique, and after many determinations it became apparent that these two bones from rats receiving a rickets-producing diet showed a difference in the degree of calcification, and that this difference increased with an increase in the severity of the rickets. Coward, Kassner & Waller [1938] have published a series of bone ash values from rats receiving a diet deficient in calcium. These values were all much lower than normal even with ample supplements of vitamin D. Their results show that in all groups the percentage ash was at a level usually associated with rickets but that there was little difference between humerus and femur. To study this point further a detailed analysis of all bone ash values which had been obtained in this laboratory was undertaken.

COLLECTION OF DATA

Data have been obtained over a period of several years, and include results already published. The percentage ash in the dry fat-free humerus was chosen arbitrarily as the basal value, and the results have been arranged in order of magnitude with respect to this datum, irrespective of sex of the rat, and of the dose of vitamin D given. The difference (d) between the percentage ash in the humerus and femur was related to the percentage ash in the humerus (H) by means of the expression $(d \times 100)/H$.

EXPERIMENTAL

All the animals were albino rats from the inbred colony of Wistar strain, maintained on a stock diet [Coward, 1938]. The young rats were weaned at 23 days, at an average weight of 40–45 g.; the test period was 5 or 6 weeks, except in one early experiment of only 4½ weeks; four rats out of the total of 38 on stock diet were fully grown. At the end of the test period, both humeri and both femora were dissected out and the ash content determined on the dry fat-free bones, the right pair being taken from each rat. In two early experiments both humeri and both femora were ashed together, but this was not the usual procedure.

RESULTS

A. *The influence of diet*

Evidence was available from three different types of diet:

(*a*) Rachitogenic diet of the high Ca and low P type, Steenbock 2965 [Steenbock & Black, 1925]: 194 rats.

(*b*) Low Ca diet, the 'poorer class' diet described by Coward *et al.* [1938]: 109 rats.

(*c*) Stock diet as used in the rat colony: 38 rats.

The results, which for convenience have been grouped in ranges of four according to the percentage ash of the humerus, are shown in Table 1 and represented graphically in Fig. 1. The rats receiving stock diet, being so few in number, have been included in a single group in the table. In severe rickets the difference in calcification between humerus and femur is clearly shown. This difference decreases as the state of calcification improves. On the other hand, there is no difference between the two bones of rats receiving either stock diet or low Ca diet.

All the evidence available related to differences between humerus and femur from rats which had received the rachitogenic diet + vitamin D, so an experiment was carried out to determine the effect of the unsupplemented diet. It has been shown [Bourdillon, Bruce, Fischmann & Webster, 1931; Bacharach, 1940; Coward & Kassner, 1941] that the variation between litters is greater than the variation within the litter for many types of biological response. In the figures so far quoted no account has been taken of litter-mates nor indeed of any variable other than the percentage ash in the dry fat-free humerus. Therefore in this experiment comparisons were made of litter-mates subjected to the different conditions.

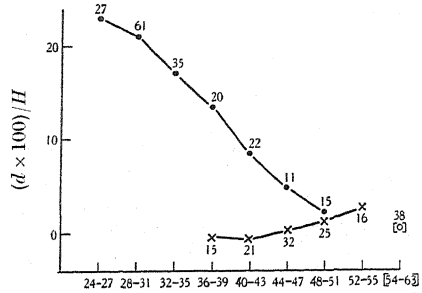

Fig. 1. The relation between the ash content of the humerus and the difference in calcification of humerus and femur of rats receiving three types of diet. •—• Rachitogenic diet. ×—× Low calcium diet. [o] Stock diet. Small figures give the number of animals.

39 rats were divided into three litter-mate groups of six bucks and seven does. Group 1 received stock diet; group 2 received the rachitogenic diet with 80 i.u. vitamin D_2 per week, this being a dose greatly in excess of the prophylactic requirement for young rats of our colony; group 3 received the rachitogenic diet only.

As comparatively few figures are available in the literature for individual responses in bone calcification, full protocols as well as the average values for this test are presented in Table 2. The regularity of response in these three groups, where maximum effects are being measured, is the outstanding feature of this table. The range of variation in the percentage ash of the humerus in all three groups is lower than that usually found among rats receiving the rachitogenic diet with a supplement of vitamin D insufficient to give complete calcification. Particularly is this the case for group 3, for under these conditions of diet rickets develops with regularity.

In the rachitic bones from group 3 in which the calcification is very poor (28·11 % ash in the humerus), the difference between humerus and femur is striking and is clearly significant. On a complete diet there is a difference in calcification between the humerus and femur, $t_d = 2{\cdot}10$, P = very slightly $> 0{\cdot}05$ for group 1. The calcification in group 2 is less than that in group 1, and the difference between the humerus and femur, though small, is significant, $t_d = 3{\cdot}55$, P = much $< 0{\cdot}01$. The inability of vitamin D alone to correct the faulty calcification brought about by a rachitogenic diet of the high Ca and low P type has often been recognized and is generally attributed to the limiting effect of the P [Hume, Pickersgill & Gaffikin, 1932].

It should also be noted that when the ash is calculated on the basis of mg./100 g. body weight

Table 1. *The absolute and percentage differences in ash content of humerus and femur of rats receiving three types of diet and the standard deviations of those differences*

Range of % ash in the humerus	Mean difference 'd' between the % ash in the humerus and femur			Standard deviation of 'd'			Mean difference 'd' expressed as % $(d \times 100)/H$			Standard deviation of $(d \times 100)/H$		
	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet
20–23	(3·04)*	—	—	—	—	—	(13·5)*	—	—	—	—	—
24–27	6·22	—	—	1·38	—	—	23·0	—	—	5·08	—	—
28–31	6·29	—	—	1·77	—	—	20·9	—	—	5·85	—	—
32–35	5·77	—	—	2·09	—	—	17·0	—	—	6·17	—	—
36–39	5·06	−0·09	—	2·39	1·69	—	13·4	−0·3	—	6·32	4·41	—
40–43	3·49	−0·24	—	2·40	2·06	—	8·4	−0·5	—	5·81	4·93	—
44–47	2·25	+0·15	—	2·88	1·26	—	4·9	0·3	—	6·35	2·70	—
48–51	1·09	+0·58	—	1·33	1·42	—	2·2	1·2	—	2·69	2·81	—
52–55	(0·63)†	+1·47	—	—	1·14	—	(1·2)†	2·7	—	—	2·06	—
54–63	—	—	0·11	—	—	0·79	—	—	0·2	—	—	1·36

* Two rats only. † One rat only.

The number of animals in each group varies from 11 to 61 and is given in Fig. 1.

the calcification in the group receiving the rachitogenic diet alone is very much less (54·4 mg. ash/100 g.) than that of the rats receiving either stock diet (130·8 mg. ash/100 g.) or the rachitogenic

Table 2. *Full protocols of individual responses as regards bone ash and body weight of rats receiving stock diet or rachitogenic diet with and without added vitamin D_2*

Group	Diet	Litter	Sex	% ash in humerus H	Difference in % ash between humerus and femur d	Difference expressed as % $(d \times 100)/H$	Body weight: At start g.	Body weight: At end g.	Increase in wt. g.	Total ash from both bones expressed as mg./100 g. body weight
1	Stock	9700	♀	56·40	−0·24	−0·4	42	111	69	141·4
		9702	♂	57·28	0·70	1·2	45	173	128	120·3
			♀	55·91	−0·72	−1·3	46	124	78	145·1
		9705	♀	57·14	−0·23	−0·4	46	138	92	139·6
		9715	♂	56·52	−0·06	−0·1	46	155	109	119·5
			♀	59·06	1·23	2·1	44	144	100	141·0
		9720	♂	54·50	0·74	1·4	48	153	105	121·4
			♀	57·85	1·18	2·0	42	131	89	129·1
		9722	♂	57·50	1·49	2·6	46	158	112	125·5
			♂	58·33	0·04	0·1	48	175	127	122·7
		9728	♀	58·65	0·78	1·3	46	127	81	134·3
		9744	♂	57·41	2·58	4·5	48	180	132	122·7
			♀	57·14	−0·45	−0·8	41	128	87	138·2
		Average		57·21	0·54	0·94	45·2	145·9	100·7	130·8
		S.D.		1·20	0·93	1·63	—	—	—	9·36
2	Rachitogenic plus excess vitamin D_2	9700	♀	51·33	1·80	3·5	46	77	31	118·7
		9702	♂	49·78	0·66	1·3	49	83	34	122·8
			♀	51·45	0·87	1·7	44	84	40	124·9
		9705	♂	50·63	0·40	0·8	50	98	48	119·1
		9715	♂	50·13	0·86	1·7	50	107	57	105·6
			♀	49·75	1·67	3·4	43	71	28	113·0
		9720	♂	49·83	4·55	9·1	45	70	25	109·6
			♀	49·34	2·65	5·4	47	66	19	127·9
		9722	♂	49·06	0·55	1·1	47	96	49	105·0
			♀	52·73	0·63	1·2	45	83	38	121·2
		9728	♀	51·92	1·19	2·3	44	59	15	144·7
		9744	♂	48·64	1·12	2·3	49	70	21	126·9
			♀	48·40	−0·69	−1·4	41	64	23	122·7
		Average		50·23	1·25	2·49	46·2	79·1	32·9	120·2
		S.D.		1·31	1·27	2·55	—	—	—	10·62
3	Rachitogenic	9700	♀	27·37	8·91	32·6	43	54	11	48·3
		9702	♂	28·50	6·91	24·2	50	68	18	52·2
			♀	27·57	4·42	16·0	43	56	13	56·4
		9705	♂	27·16	6·83	25·1	45	53	8	60·6
		9715	♂	28·15	6·86	24·4	45	53	8	57·0
			♀	28·75	5·58	19·4	44	52	8	57·9
		9720	♂	27·92	6·95	24·9	48	63	15	54·9
			♀	29·08	5·74	19·7	46	56	10	55·5
		9722	♂	29·25	7·31	25·0	48	63	15	51·6
			♀	27·64	5·17	18·7	46	61	15	53·1
		9728	♀	29·43	7·21	24·5	46	59	13	57·1
		9744	♂	28·21	8·92	31·6	49	65	16	51·1
			♀	26·39	6·99	26·5	41	51	10	51·8
		Average		28·11	6·75	24·05	45·7	58·0	12·3	54·4
		S.D.		0·89	1·30	4·77	—	—	—	3·41

diet + vitamin D (120·2 mg. ash/100 g.). The increase in body-weight is considerably less for rats on the rachitogenic diet than for rats on stock diet, even with excess of vitamin D. On the rachitogenic diet alone, this increase was very small from the start, and during the last 2 weeks a slight decrease took place.

B. *Distribution of calcification in the bone*

In the rachitic bone the metaphyses are greatly affected while the changes in the shaft are smaller and proceed at a slower rate during the development of the lesions [Hess, Berliner & Weinstock, 1931]. This suggested that the relatively small total ash content of the femur of a rachitic rat might be due entirely to the relatively large metaphyses. The weights and ash contents of the ends and shaft of the bones were therefore determined separately.

Left bones representative of each of the three diets (stock diet, low Ca diet, rachitogenic diet) were taken. The division of each bone into three, a shaft and two ends, was chosen arbitrarily but the cuts were always made as nearly as possible in the same places—in the femur just below the lesser trochanter and just above the condyles, in the humerus at the surgical neck and just below the supratrochlear fossa. For each bone the shaft was weighed alone and the two ends together. The results are shown in Table 3. Calcification is distributed in approximately similar proportions between shaft and ends in both humerus and femur for groups 2, 3 and 4; that is to say for rachitogenic diet + vitamin D_2, low Ca diet and stock diet, the humerus and femur show about the same degree of calcification, although the level varies with the diet, and so do the ends of both humerus and femur. In contrast to this, these bones from rats which have received the unsupplemented rachitogenic diet differ from one another both as regards the distribution of calcification between shaft and ends and also as regards absolute values for ash contents. The figures for rachitic rats show a difference between the two bones even when the shafts and ends are considered separately. The relatively low ash content of the whole femur was thus not entirely due to the fact that its metaphyses were relatively large, though it was partly due to this factor since the percentage difference was larger for the whole bone (6·68) than for either the shaft (4·60) or ends (4·27).

Table 3. *Ash content of ends as compared with whole bone and comparison between these portions in femur and humerus*

Group	No. of animals	Diet	Weight of ends / Total weight × 100		% ash in whole bone (calculated)		Difference in % ash in humerus and femur		
			Humerus	Femur	Humerus	Femur	Shaft	Ends	Whole bone
1	8	Rachitogenic	51	61	29·40	22·72	4·60	4·27	6·68
2	6	Rachitogenic + vitamin D	47	49	49·82	48·23	2·66	−0·27	1·59
3	10	Low Ca	46	45	46·70	45·61	2·87	−0·88	1·09
4	7	Stock	42	41	57·85	56·19	2·75	0·36	1·66

The fall in the ash content of the bones of the rachitic rats was most marked in the femur and particularly in the ends of the femur. This is perhaps due to the strain placed upon this bone because the weight of the rat is carried mostly on its hind legs.

In view of the many sources of possible error both in cutting dry fat-free bones and in ashing very small pieces of bone it should be emphasized that the figures in Table 3 represent only a general state of affairs. When the percentage ash in the whole left bone was calculated from that of the two parts and compared with the percentage ash previously determined for the corresponding right bone, the agreement in nearly all cases was good, showing that the figures in the table are representative of the bone both as regards relative proportion of parts and as regards their calcification.

These results suggest that, as is the practice in both the X-ray and the line test techniques, one part of one bone only might, with advantage, be used in the assay of vitamin D by the bone ash method. The difference in ash content between a severely rachitic bone and one showing the maximum calcification brought about by vitamin D alone, is 19·89 % for the humerus alone, 26·06 % for the femur alone, 22·98 % for the average of the humerus and femur and 28·31 % for the ends of the femur alone. These figures suggest that by using for the assay only the ends of the femur, which show the large response to vitamin D, an increase in the range of calcification is secured and hence a steeper curve of response and one which might be more sensitive for low doses. But further work is required, since the data here reported are insufficient to establish the point.

C. *Influence of sex*

In the young female rat ossification proceeds more rapidly than in the male [Cox & Imboden, 1936]. Donaldson [1924] gives figures showing that

Table 4. *The influence of sex on bone ash content*

Diet	Range of % ash in the humerus	No. of animals ♂	No. of animals ♀	Average value of d ♂	Average value of d ♀	Average value of $(d \times 100)/H$ ♂	Average value of $(d \times 100)/H$ ♀
Rachitogenic	24–27	18	9	6·29	6·07	23·4	22·4
	28–31	35	26	6·49	6·02	21·7	19·9
	32–35	20	15	5·89	5·60	17·4	16·4
	36–39	12	8	4·38	6·07	11·7	16·0
	40–43	10	12	3·16	3·77	7·6	9·1
	44–47	3	8	4·58	1·38	9·7	3·0
	48–51	7	8	1·09	1·09	2·2	2·2
Low Ca	36–39	10	5	0·12	−0·50	0·3	−1·4
	40–43	8	13	−0·43	−0·12	−1·1	−0·2
	44–47	20	12	0·38	−0·24	0·8	−0·5
	48–51	17	8	0·82	0·08	1·6	0·2
	52–55	6	10	1·34	1·55	2·5	2·9
Stock	54–62	20	18	0·17	0·05	0·3	0·1

at both 100 and 150 days of age the humerus and femur of female rats have a higher percentage of ash than those of male rats. The figures given in Table 1 have been analysed in Table 4 according to the sex of the animal. The differences in ash content between the rachitic humerus and femur is shown equally by both sexes.

SUMMARY

1. When low calcification in rats, as measured by the ash content of the humerus, is due to shortage of vitamin D, the femur shows an even lower calcification. When, however, it is due to a deficiency of either Ca or P without a simultaneous shortage of vitamin D in the diet, no difference in calcification between the two bones is observed, neither is a difference found in the bones of rats receiving a stock diet.

2. In the femur both the relatively large metaphysis and the extremely low calcification of this part make the ash content of the whole bone lower than that of the humerus when there is a deficiency of vitamin D in the diet.

3. The differences between the rachitic humerus and femur are not influenced by the sex of the animal.

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The Preparation of Deoxycholic Acid from Cholic Acid

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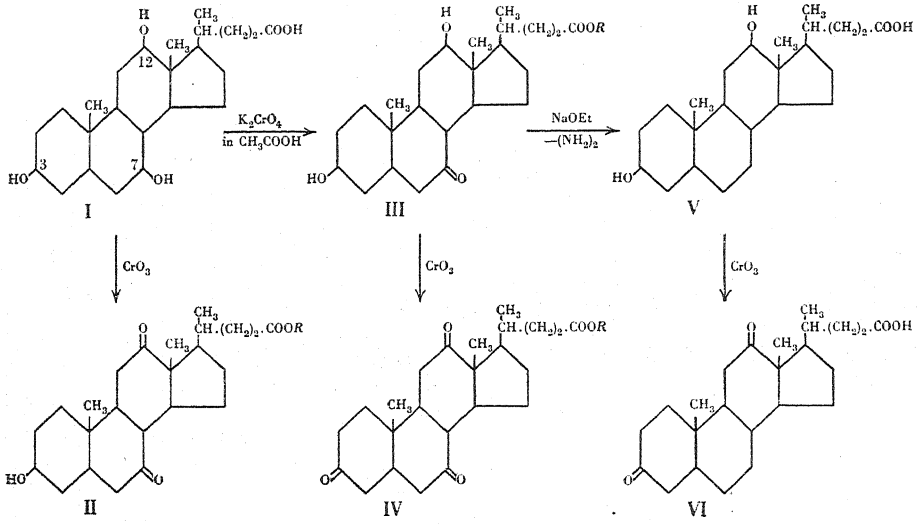
Deoxycholic acid (3:12-dihydroxycholanic acid) has been made from cholic acid in small yield by hydrogenation of the derived 3:12-dihydroxycholenic acid [Boedecker & Volk, 1922], but this method is of academic interest only. The present work, as already stated [Haslewood, 1942], aims at the production of deoxycholic acid by a convenient method from the readily available cholic acid, to supplement the

rather uncertain preparation from bile of Na deoxycholate for bacteriological use. Kaziro & Shimada [1937] showed that cold chromic oxidation of cholic acid (I) gave 3-hydroxy-7:12-diketocholanic acid (II, $R=H$): this was confirmed by Bergström & Haslewood [1939]. Moreover, although Kaziro & Shimada could not obtain a mono-ketonic acid, they indicated that such a substance might be formed, in an overall yield of about 30 % of theory. Deoxycholic acid, also of good quality and apparently free from cholic acid, was given in an overall yield of 40–50 % of theory by direct reduction of the crude oxidation product. Lithocholic acid may have been an impurity in this final material, for, although it was not apparent therein, it was found in small amount in the mother-liquors. Reduction



since Wieland & Dane [1932] had shown that 7:12-dihydroxycholanic acid was oxidizable to give a 7-keto-12-hydroxy derivative. Direct chromic oxidation of cholic acid has now been made to yield the expected derivatives of 7-keto-3:12-dihydroxycholanic acid (III), easily reduced to deoxycholic acid (V). This, isolated as the ether-complex, was identified by its usual properties and by conversion into the characteristic dehydrodeoxycholic acid (VI).

Controlled chromic oxidation was carried out by the addition of K_2CrO_4 to cholic acid dissolved in acetic acid. Many experiments showed that the highest yield of 7-keto-compounds was obtained (*a*) when the solvent was highly purified acetic acid, and (*b*) when the reaction was greatly slowed. These conditions were met by the addition of measured amounts of K_2CrO_4 in concentrated solution and by buffering the mixture with Na acetate. This method enabled a crystalline semicarbazone (mainly of 7-keto-3:12-dihydroxycholanic acid III, $R=H$) to be isolated in yields of about 45 % of theory when chromate was added in amount equivalent to one atom of oxygen. The semicarbazone, however, contained small amounts of cholic acid, which appeared in the final product. This impurity could be removed, but better yields were obtained by using chromate equivalent to two atoms of oxygen, when the semicarbazone, though contaminated with high-melting material, gave a very pure deoxycholic acid [cf. Longwell & Wintersteiner, 1940; Kon & Soper, 1940] was performed with Na ethylate-hydrazine at 200° for 3 hr., with the use of minimal amounts of Na.

The intermediates (III), 7-keto-3:12-dihydroxycholanic acid and its ethyl ester, were obtained from the oxidation product or from the semicarbazone. On acid-alcoholic hydrolysis, the latter gave *ethyl 7-keto-3:12-dihydroxycholanate* (III, $R=C_2H_5$), M.P. (uncorr.) 160–161°, depressed by ethyl 3-hydroxy-7:12-diketocholanate (II, $R=C_2H_5$) but not by ethyl cholate. The same ester M.P. 160–161° was isolated after esterification of the crude chromic oxidation product. It is easier to prepare the 7-keto-ester (the most easily purified of the intermediates) by over-, rather than by under-oxidation of cholic acid, since its separation from ethyl cholate is difficult. The crystalline ester (III, $R=C_2H_5$) was oxidized to ethyl dehydrocholate (IV, $R=C_2H_5$) and reduced, with saponification, to deoxycholic acid. The interesting *7-keto-3:12-dihydroxycholanic acid* (III, $R=H$), from the ethyl ester, separated from dilute aqueous acid as hydrated crystals, M.P. *ca.* 83°, and could be recrystallized from the same solvent although it did not crystallize from organic solvents in the usual way. The cholic acid derivatives could be distinguished from the others here mentioned by the HCl test of Hammarsten [1909], which gave a yellow colour with the 7-keto-compounds (III) and

green colours with mixtures of these and substances with the cholic acid nucleus. The test was of great value during this investigation.

EXPERIMENTAL

All M.P. are uncorrected. Elementary micro-analysis was carried out by Dr H. Nisbet, Edinburgh. Deoxycholic acid was obtained as the ether-complex, M.P. 150–155°. Samples of every preparation obtained in altered conditions were oxidized in the usual way with CrO_3 to give dehydrodeoxycholic acid, crystallizing from dilute alcohol in characteristic glistening leaflets of M.P. 184–186°, decomp., not depressed by authentic material.

Hammarsten's HCl test was done by boiling a mixture of 2–3 mg. of material with 0·2 ml. of strong HCl in a small tube for 1–2 min. The characteristic colours appeared after 1–2 hr.

Preparation of deoxycholic acid. (*a*) *Oxidation and purification.* *n* g. ($n = 1$–10) of dried and powdered cholic acid were dissolved at 60–70°, in a flask of at least 100*n* ml. capacity, in 10*n* ml. of glacial acetic acid. At the same temperature, 2*n* g. of $CH_3COONa.3H_2O$ crystals were added and dissolved. The mixture was cooled at once to 18–20° and treated with 2*n* ml. ($\equiv 2n$ atoms of O) of K_2CrO_4 solution (31·7 g. of K_2CrO_4/100 ml.), added slowly with shaking, agitation at intervals being continued until all precipitated chromate had dissolved. After at least 24 hr., the solution was diluted with 70*n* ml. of H_2O, and 10*n* g. of crude NaCl were added. When this had dissolved, and after further standing, with occasional shaking, for 24 hr., the liquor was decanted through a filter and the residue and filter washed with H_2O. 10*n* ml. of *N* NaOH followed by 20*n* ml. of H_2O were poured through the filter and back into the original flask. This was now heated, with agitation, until the contents dissolved and the solution gently boiled. After 5–10 min. boiling and when Cr compounds had coagulated, the flask was partially cooled and the still warm mixture treated with 5*n* g. of crude NaCl, dissolved with shaking, followed by 20*n* ml. of *N* H_2SO_4. The precipitate was collected after 16 hr. and well washed on the filter with cold H_2O. It was then allowed to become partially dry, and dissolved in cold alcohol. The filtered solution was evaporated, finally at the pump, leaving 0·8–0·9*n* g. of a yellowish gum (*product A*) giving a yellow colour in the Hammarsten test.

(*b*) *Reduction.* 5 ml. of an ethyl alcoholic solution containing 40 g./100 ml. of *product A* were added to a metal bomb containing a solution of 0·4 g. Na in 5 ml. ethyl alcohol, with 2 ml. of hydrazine hydrate (95/98%). The mixture was carefully heated almost to boiling; then the bomb was sealed and heated at 200–210° for 3 hr. An aqueous extract (*ca.* 200 ml.) of the contents of the cooled bomb was acidified with dilute H_2SO_4, the precipitate being warmed gently to disperse gels. After 2–3 hr., the white solid was collected, washed with water and dissolved in cold alcohol. Evaporation of the filtered solution left a gum which crystallized at once when about 10 ml. of ether were added. After warming with the ether, the product was allowed to stand for several hours with occasional shaking, and was finally cooled to 0°. The crystals were collected (suction), washed with ice-cold ether, and dried. Yield, 1·1–1·2 g.; small white needles M.P. 150–155°, HCl-test, faint yellow-green. 0·2 g. of this substance, from 2–3 ml. of acetic acid, gave 0·17 g. of white needles M.P. 139–141°.

4 g. of residue obtained on evaporation of the ether mother liquors from (*b*) were dissolved in a little fresh ether. During 24 hr., crystals accumulated. The liquor was decanted and the crystals washed with ether and cold alcohol, collected, and dried. This material (0·15 g.) had M.P. 184–186°, not depressed by lithocholic acid; it was not obviously affected by HCl.

Semicarbazone. To *product A*, from *n* g. of cholic acid, were added *n*/2 g. of semicarbazide hydrochloride, *n*/2 g. of Na acetate crystals, and 5*n* ml. of 50% (by vol.) alcohol/water. This mixture was warmed till all had dissolved, boiled under reflux for 1 hr. and allowed to stand 3–5 days. The solid was collected (suction), washed with 50% alcohol, water, alcohol, and ether, and dried at 60–80°. Yield, 0·6*n* g., M.P. >330°; HCl-test, yellow. (Found, N, 11·2%.) When the oxidation (*a*) was done with 1·1*n* ml. of K_2CrO_4 solution ($\equiv 1{\cdot}1n$ atoms of O), the semicarbazone (0·5*n* g.), prepared as described, formed groups of white needles, M.P. 250° (decomp.), which gave a blue HCl-test as also did the deoxycholic acid and 7-keto-derivatives from them. (Found, N, 8·6%.) 0·5 g. of this latter product, after boiling with 5 ml. of alcohol under reflux for 30 min. left 0·32 g. of undissolved crystals, M.P. 265°, decomp.; HCl-test, yellow-green. (Found, N, 10·0%. $C_{25}H_{41}O_5N_3$ requires N, 9·1%.) A product of M.P. 267°, decomp.; HCl-test, green, was given when $K_2CrO_4 \equiv 1{\cdot}5n$ atoms of O was used for the oxidation (*a*).

The above semicarbazones could be reduced by heating (in a sealed bomb at 200–210° for 3 hr.) 1 g. samples with a solution of 0·25 g. of Na in 10 ml. ethyl alcohol, with 1 ml. of hydrazine hydrate. The deoxycholic acid, obtained as under (*b*), had similar properties, that from the semicarbazone M.P. >330° appearing to be of especially good quality. Overall yield of deoxycholic acid via the semicarbazone, about 30% of theory.

Ethyl 7-keto-3:12-dihydroxycholanate. (i) 6·5 g. of *product A* were boiled for 45 min. under reflux with 13 ml. of a mixture of ethyl alcohol (20 ml.) and concentrated H_2SO_4 (5 ml.). The cooled solution was diluted with water and extracted three times with ether. The ether, washed with water and Na_2CO_3 solution, was evaporated, and the residue crystallized from methyl alcohol; 1·6 g. of crude crystals were obtained.

(ii) 3 g. of semicarbazone M.P. >330° were boiled under reflux for 1 hr. with 30 ml. of a mixture of ethyl alcohol (40 ml.), H_2O (10 ml.), and conc. H_2SO_4 (5 ml.). From the cooled and diluted solution, crude crystals (0·9 g.) were obtained exactly as under (i). The ester crystallized from methyl alcohol in large colourless rectangular plates or prisms, which, after drying at 70–80°, had M.P. 160–161°, not depressed by ethyl cholate. (Found, C, 72·0; H, 9·9%. $C_{26}H_{42}O_5$ requires C, 71·9; H, 9·7%.) The compound formed glistening leaflets from a diluted methyl alcoholic solution: it gave a yellow colour with strong HCl.

A purified sample of ethyl 7-keto-3:12-dihydroxycholanate was oxidized in the usual way with CrO_3 to ethyl dehydrocholate, M.P. 222–223° (decomp.), and another sample was reduced as under 'Semicarbazone' to deoxycholic acid (0·13 g. from 0·2 g. of ester, M.P. 160–161°). By the same process, 0·2 g. of ethyl cholate gave 0·14 g. of cholic acid.

7-keto-3:12-dihydroxycholanic acid. 0·3 g. of the above ester, M.P. 160–161°, was boiled under reflux for 30 min. with a mixture of Na (70 mg.), ethyl alcohol (1·5 ml.), and H_2O (1 drop), the ester being added after the Na had all dissolved. After dilution the cold solution was filtered, further diluted to 200 ml., heated to boiling and acidified with

5 ml. of N H_2SO_4. After 3–4 days, the white needles which had formed were collected and dried *in vacuo*. Yield, 0·13 g., M.P. 83°, giving a yellow colour with concentrated HCl and in the Liebermann-Burchard test, and a red colour with concentrated H_2SO_4. There was difficulty in removing solvent from this substance for analysis. (Found: C, 70·8; H, 9·5%. M (titration), 395. $C_{24}H_{38}O_5$ requires C, 70·9; H, 9·4%; M, 406.) The acid could be recrystallized by acidification of a hot alkaline solution as described, but did not form crystals from organic solvents. It dissolved easily in alcohol, acetone, and acetic acid, and to some extent in ether, but appeared to be insoluble in benzene and light petroleum.

SUMMARY

1. By a simple three-stage process (oxidation, purification, reduction), cholic acid has been converted into deoxycholic acid, with an overall yield of 40–50% of theoretical.

2. Preferential oxidation of cholic acid at C_7 was performed by adding measured amounts of concentrated K_2CrO_4 solution to cholic acid dissolved in acetic acid buffered with Na acetate.

3. The partially purified product was then reduced with Na ethylate-hydrazine, yielding deoxycholic acid. Deoxycholic acid was also prepared from an impure semicarbazone, the preparation and properties of which were used to follow and define the conditions of the oxidation. The intermediate 7-keto-3:12-dihydroxycholanic acid and its ethyl ester have been isolated and examined.

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Dental Depigmentation in the Rat

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In normal rats the anterior surfaces of the incisor teeth are a rich brown or orange. According to Marshall [1921] the pigment is confined to the outer fibrous layer. The whitening of the teeth which occurs in vitamin A deficiency has been described by Wolbach & Howe [1925; 1933], Smith & Lantz [1933], Schour, Smith & Hoffman [1938], Schour, Hoffman & Smith [1941], Irving & Richards [1939] and H. Mellanby [1939]. In recent experiments on the interaction of vitamin A and vitamin E, Davies & Moore [1941] found that under certain circumstances whitening of the teeth may result from the exclusion of vitamin E from the diet. It was suggested that this lesion might be caused by deficiency of either vitamin. Evidence in favour of this view is given in the present communication.

EXPERIMENTAL

Grading of depigmentation. To assess the degree of depigmentation in individual rats each tooth was awarded marks according to the following arbitrary scale.

Description	Marks given
White	25
Faint brown mottling on white	20
Cream	15
Mottled brown and white	10
Deep cream	10
Light brown	5
Brown	0

Thus the dental depigmentation of a rat with four completely white teeth is expressed by 100 marks, while four completely brown teeth are indicated by 0 marks.

The cure of white teeth in rats deficient in vitamin A by the administration of carotene

In routine tests for vitamin A in samples of butter and margarine, male albino and piebald rats were given a basal diet of caseinogen (Glaxo, alcohol extracted) 20%, sugar 60%, arachis oil 15%, salt mixture 5% and dried yeast 10%. Vitamin E was supplied as 1 mg. weekly of *dl*-α-tocopherol acetate dissolved in arachis oil, and vitamin D as 1 drop of 'Radiostol' (B.D.H.). In addition small doses of

the butter or margarine were given to supply enough vitamin A to allow fairly rapid growth. At the end of the test 12 rats with completely white teeth were nevertheless available for use in the present investigation. These were kept on the same basal diet and divided into 4 groups, which were dosed with a solution of carotene in arachis oil at levels of 2, 6, 16 and 44 i.u. per rat daily.

The changes in weight during the next 59 days are shown in Table 1.

Table 1. *Growth of rats receiving different doses of carotene*

Daily dose of carotene i.u./day	Group 1 2			Group 2 6			Group 3 16			Group 4 44		
Rat no.	1	2	3	4	5	6	7	8	9	10	11	12
Wt. (g.) on day 1	178	173	210	160	154	143	166	148	182	126	112	187
Wt. (g.) on day 60	174	130	Died	210	197	180	219	220	222	194	193	267
Mean increase in wt. (g.)		−23			+43			+55			+76	

Signs of the return of brown colour to the teeth were seen in some rats of groups 3 and 4 after 30 days, and after 60 days most of the animals had almost normal teeth. The teeth in groups 1 and 2 remained almost completely white. The marks given are shown in Table 2.

Table 2. *Dental depigmentation in rats receiving different doses of carotene*

Daily dose of carotene i.u./day		Group 1 2			Group 2 6			Group 3 16			Group 4 44		
Rat no.		1	2	3	4	5	6	7	8	9	10	11	12
Depigmentation of teeth (arbitrary marks)	Day 1	100	100	100	100	100	100	100	100	100	100	100	100
	Day 60	75	90	Dead	90	90	80	20	20	10	10	10	45
Mean mark at end of exp.			83			87			17			22	

Examination of the unerupted portions of the teeth gave similar results.

The cure of white teeth in rats deficient in vitamin E by the administration of dl-α-*tocopherol*

Female albino rats were kept for periods of 4 or 7 months on a diet deficient in vitamin E, consisting of caseinogen ('light white') 25 %, sugar 50 %, lard 10 %, dried yeast 10 % and salt mixture 5 %. Vitamin A was supplied at the level of about 1000 i.u./week in the form of halibut liver oil. The animals, most of which showed marked dental depigmentation, were then divided into two groups, each comprising three rats which had received the deficient diet for 4 months, and four rats which had received the same diet for 7 months. One group was now given 1 mg. of tocopherol acetate weekly, the other received no vitamin E. In connexion with another experiment each group received occasional injections of lard, a treatment which would not be expected to influence the present results.

The weights of the rats at the time of division into groups, when they were already nearly full grown, and after further periods of 84 and 203 days, are given in Table 3.

In group 1 two rats died after receiving tocopherol for about 3 months. With rat no. 8 death was probably due to sepsis following the injection of lard. Rat no. 7 was not autopsied, but it was emaciated and had overgrown teeth which may have caused difficulty in eating. The data in Table 3

Table 3. *Weights of rats receiving vitamin E-deficient diet*

Group 1. Given 1 mg. of tocopherol acetate per rat, each week

Rat no. ...	13	14	15	16	17	18	19
Wt. (g.) on day 1	202	250	196	187	169	182	186
Wt. (g.) on day 84	227	227	214	245	192	203	171
Wt. (g.) on day 203	237	239	237	Dead	203	217	Dead

Mean increase of the five rats surviving 203 days = 27 g.

Group 2. Given no added tocopherol

Rat no. ...	20	21	22	23	24	25	26
Wt. (g.) on day 1	165	176	182	202	165	167	192
Wt. (g.) on day 84	187	192	197	211	177	185	197
Wt. (g.) on day 203	170	192	188	204	173	177	182

Mean increase of five rats showing greatest growth = 9 g.

show that little change in weight occurred in either group.

The degree of dental pigmentation at the beginning of the test period was less uniform in both groups than in the preceding experiment in which deficiency of vitamin A had been involved. The individual marks after various periods are shown in Table 4.

The mineral content of the teeth in avitaminosis E

Estimations of ash content and of fluorine in white and normal brown teeth were kindly made by Dr H. H. Green. The teeth examined were taken from rats which had been used in previous experiments [Davies & Moore, 1941]. The results are given in Table 5. There would therefore appear to be a marked increase of the ash content in whitened teeth. The values for F were within the normal range in both groups.

Table 4. *Dental depigmentation of rats receiving vitamin E-deficient diet*

Group 1. Given 1 mg. tocopherol acetate per rat, each week

	Rat no. ...	13	14	15	16	17	18	19	Mean
Depigmentation of teeth (arbitrary marks)	Day 1	100	0	100	70	70	100	100	76
	Day 84	0	0	0	0	0	0	100	15
	Day 203	0	0	0	Dead	0	0	Dead*	0

Group 2. Given no added tocopherol

	Rat no. ...	20	21	22	23	24	25	26	Mean
Depigmentation of teeth (arbitrary marks)	Day 1	100	90	100	90	90	90	90	93
	Day 84	100	70	90	60	70	70	65	75
	Day 203	100	50	90	90	95	90	95	87

* Teeth still white at death, but malformed and overgrown.

If we omit from consideration rat no. 14, which had brown teeth at the beginning, and also no. 19, which was diseased, the remaining five rats in group 1 all showed complete cure as the result of treatment with tocopherol. Of the seven rats in group 2 only no. 21 showed slight improvement, the teeth of the other six remained almost completely white.

At the end of the experiment the five surviving rats of group 1, and five rats of group 2, were killed and the vitamin A reserves in their livers estimated by the $SbCl_3$ method. For group 1 the mean total reserve was 14,000 i.u. For group 2, in confirmation of previous observations on the effect of vitamin E deficiency in lowering the vitamin A reserve [Moore, 1940], the mean value was only 4700 i.u., which, however, is still about twice the reserve found in a wild rat shot near this laboratory [Davies & Moore, 1934].

Table 5. *Mineral analysis of teeth from normal rats and from rats deficient in vitamin E*

Rat no.	Wt. of lower incisors g.	Dry matter %	Ash content %	Fluorine p.p.m.* original tooth
	(*a*) Normal rats (brown teeth)			
1	0·198	86·5	64·5	50
2	0·129	87·5	64·0	115
3	0·107	89·5	65·0	140
4	0·140	89·5	62·5	140
Mean	0·144	88·2	64·0	111
	(*b*) Deficient rats (white teeth)			
1	0·141	89·0	67·5	35
2	0·189	90·5	71·5	80
3	0·166	89·5	66·5	180
4	0·125	90·5	72·0	80
Mean	0·155	89·9	69·4	94

* p.p.m. = parts per million.

DISCUSSION

The above experiments suggest that whitening of the teeth in the rat results from deficiency of either vitamin A or of vitamin E, and may be cured by the restoration of whichever of these vitamins is missing. Since vitamin E influences the efficiency of storage and utilization of vitamin A [Moore, 1940; Bacharach, 1940; Davies & Moore, 1941] the occurrence of a common lesion in deficiency may imply a further aspect of their interaction. Although deficiency of vitamin E may under some circumstances cause a secondary deficiency of vitamin A, indicated by the premature depletion of the vitamin A reserves in the liver, whitening of the teeth in vitamin E deficiency may occur even when large amounts of vitamin A are given and the reserves in the liver are high. While therefore the dental lesion may possibly result from a disturbance in the function of vitamin A as a result of the absence of vitamin E, a material deficiency of vitamin A is not an essential aetiological factor.

Dental depigmentation also occurs in fluorosis [McCollum, Simmonds, Becker & Bunting, 1925] and in Mg deficiency [Duckworth, 1942]. The lesion may therefore result from various dietary abnormalities, which may or may not be interrelated. Moreover, Irving [1942] has detected lesions in the enamel-forming organs of rats deficient in vitamin E, distinct from those observed in avitaminosis A, at a stage of the deficiency when the

teeth are still brown. Depigmentation may therefore be the final outcome of different initial histological lesions.

The persistence of dental depigmentation in rats which have been deprived of vitamin A and subsequently treated with small doses of this vitamin affords an excellent demonstration of the reality of the conception of suboptimal nutrition. Rats in the group given 6 i.u. of vitamin A daily grew almost as rapidly as those given 16 i.u., but whereas 16 i.u. was sufficient to restore the brown colour of the teeth, 6 i.u. was inadequate. Ability to sustain rapid growth does not therefore necessarily imply that the animal is receiving the full supply of nutrients necessary for perfect health.

SUMMARY

1. Whitening of the teeth in rats was induced by deficiency of either vitamin A or vitamin E, and could be cured by restoration of whichever vitamin was missing.

2. A low dose of vitamin A, which was sufficient to restore rapid growth in rats previously deprived of this vitamin, was ineffective in restoring the normal brown colour of the teeth.

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The Microbiological Degradation of Steroids

1. THE STEROL CONTENT OF SOILS

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The wide distribution of free or esterified sterols in animal and vegetable tissues must result, during the decay of such organic material, in the deposition in soils of an appreciable quantity of various sterol substances. Schreiner & Shorey [1909; 1911] have reported the isolation from soils of two sterols. The first of these, agrosterol, was present in the free condition, and was considered to be the sterol component of some plant tissue, or to have originated by structural alteration from some substance of entirely different composition present in the soil. The second, phytosterol, M.P. 135°, occurred only in the form of its esters, which were regarded as plant residues remaining unchanged in the particular peaty soil under examination. In general, however, the sterol content of soils seems to have received but little attention. The investigation of the various factors influencing the accumulation of sterols in, and their disappearance from, different types of soil forms the basis of the present work.

METHODS

The quantitative determination of soil sterols is attended by considerable difficulty. Humus material may be present in proportions of from less than 1 % in the largely mineral soils to almost 100 % in pure peat, and the purification of the sterols thus becomes lengthy and tedious. It has been found that a preliminary solvent treatment of the soil, followed by purification of the crude sterol extracts by means of the insoluble digitonides, yields solutions sufficiently pure for colorimetric estimation.

The soil sample is thoroughly dried in an air oven at 80°, and, after being ground in a mortar, is passed through a 30-mesh sieve to remove pebbles, plant fragments, etc. 1 kg. of soil obtained in this way is extracted continuously with acetone. After removal of the solvent by distillation, the brownish gummy residue is refluxed with a slight excess of alcoholic KOH for 30 min. The solvent is removed on a water bath, and the dark-coloured viscous liquid remaining taken up in a large excess of anhyd. K_2SO_4. This solid is

extracted for 4 hr. in a Soxhlet apparatus with petroleum, B.P. 40–50°, and the extract made up to known volume. The determination of sterol in this solution is based upon the method of Kelsey [1939]. A portion containing approximately 0·5 mg. sterol is evaporated to 0·5 ml. in a 15 ml. graduated centrifuge tube. 4·0 ml. of a 0·2% solution of digitonin in 95% alcohol are added, and the mixture evaporated to dryness. The solid residue is boiled with 12 ml. petroleum, B.P. 40–50°, the tube and contents centrifuged, and the supernatant yellowish liquid discarded. This is repeated until the extracts are no longer coloured. The residual solid is then gently refluxed with 7·0 ml. benzene for 45 min. to decompose the digitonide, and after being concentrated to 3 ml., the contents of the tube are finally diluted to 12 ml. with petroleum, B.P. 40–50°. Digitonin separates on thorough stirring, and is centrifuged off. The supernatant liquid is decanted, and the residue re-extracted three times with light petroleum. The combined extracts are evaporated to dryness, and the residue taken up in 5·0 ml. $CHCl_3$. This solution is transferred to a 10 ml. glass-stoppered cylinder. 0·5 mg. cholesterol dissolved in 5·0 ml. $CHCl_3$ is measured into a second cylinder, and 1·0 ml. acetic anhydride and 0·1 ml. conc. H_2SO_4 are added to each. The solutions are thoroughly mixed, and are allowed to stand in a bath at 24° for 15 min.; the colours which have developed are then compared in a colorimeter.

Neither the digitonin precipitation used as the final purification in the above method, nor the Liebermann-Burchard colour reaction, is itself specific for sterols. Digitonin precipitation in the steroid group appears [Windaus, 1909] to depend upon a free —OH at C_3 in the cholane nucleus, and the *cis*-position of this —OH to the —Me at C_{10}. Thus, insoluble digitonides are also given by several bile acids, certain cholane cardiac poisons and their aglycones, and by various other compounds. The cardiac glycosides, in common with the saponins, many bile acids and other substances, also give the Liebermann-Burchard reaction for sterols. During the various purification stages described these compounds are eliminated, with the possible exception of certain cardiac aglycones which give both digitonin precipitates and positive colour reactions. These compounds, however, are probably far rarer in soils than the sterols, and no evidence of their presence in any of the final solutions has been obtained with the Legal nitroprusside test. The H_2SO_4-acetic anhydride colour may thus be regarded as indicative of the total sterol content of the soil, irrespective of whether the sterol was originally present in the free state, as its fatty acid esters, or in the form of its glycosides. No attempt has been made to identify individual sterols, the result in all cases being expressed in terms of cholesterol.

The accuracy of the method has been tested by determining on a number of soils the percentage recovery of cholesterol added to dried, sieved soil.

Table 1. *Recovery of added cholesterol*

Initial sterol content mg./kg. soil	Cholesterol added mg.	Total sterols by analysis mg./kg. soil	Cholesterol recovered mg.	Recovery %
1·8	61·3	61·5	59·7	97·4
0·9	52·4	54·7	53·8	102·7
20·1	25·7	45·2	25·1	97·7
Nil	18·9	18·1	18·1	95·7

The difference in the figures obtained in four experiments with the original soil and that containing added cholesterol indicates the recovery of cholesterol (Table 1). The recoveries have been of a consistently high order.

DISCUSSION

Distribution of sterols in British soils

The soils examined in this survey have been collected from widely distributed areas throughout the British Isles, and include the majority of soil types found in this region. They have, in general, been taken from a depth of from 2 to 6 in., after removal of the surface layer of living or dead vegetation, but in certain instances where the profile showed deep peaty humus layers, sampling has been carried out at a depth of several feet.

Table 2. *Sterol contents of British soils*

Vegetative type	Source	Soil type	No. of samples	Sterol substances present mg./kg. soil
Forest land	Oak and oak-birch associations (Sherwood Forest)	Coarse acid sand	6	0·6– 1·4
	Oak (Weald)	Light loam to heavy clay	2	0·1, 0·8
	Beech (Sherwood Forest, Epping Forest, Chiltern plateau)	Coarse acid sand	5	0·2– 1·2
	Beech (South Downs)	Shallow, alkaline, light loam	2	0·8, 2·5
	Beech (Chiltern escarpment)	Strongly alkaline marl	1	3·2
Grass land	Acid grassland (southern Pennines, Malvern Hills)	Acid sand or loam	2	Nil, 0·8
	Neutral grassland (Trent and Thames valleys)	Chiefly loams	4	0·4– 1·5
	Basic grassland (Chiltern escarpment)	Shallow, alkaline, marls	3	0·6– 2·7
Fen land	Wicken Fen, Cambridge, Norfolk Broads	Black, slightly alkaline peat	4	2·3– 8·5
Bog land	Peat moors, Athlone, Spring moors, New Forest, Dartmoor	Deep, acid peat	5	7·9–12·7
Heath land	Sherwood Forest, Pennine moors, Yorkshire moors, Central Scottish highlands	Coarse sand	6	Nil– 1·1
Arable land	Various	Various	12	Nil– 6·3

The results (Table 2) show clearly that definite relationships exist between sterol content and soil type. Deposition of sterols must depend primarily upon the nature and number of the animal and plant inhabitants, so that deposition will be at a maximum in soils favourable to the maintenance of a flourishing population. A characteristic of such soils, however, is the rapidity with which humus material undergoes disintegration. Since the sterol content of these soils shows no continued increase, this component of the humus is presumably decomposed or disposed of in some other manner. In soils of the opposite type there is a marked accumulation of sterols. Extreme acidity, lack of aeration, and excessive water content are all factors which, either individually or collectively, inhibit the decomposition of humus and its constituent sterols, and are thus directly responsible for the high sterol contents of bog and fen peats. In arable soils these adverse factors have been counteracted, and, whilst it is found that sterols are present in varying amount, the content of any particular soil shows a gradual decrease unless replenished by organic manuring.

Agencies responsible for sterol disappearance

In view of the insolubility of the sterols, and of their association with natural fats and oils, their disappearance from soils cannot be attributed to the leaching action of rain-water, but is probably the result of degradation of the sterol molecule by the co-ordinated activities of a variety of soil micro-organisms. Evidence in favour of this theory has been obtained experimentally in the following manner.

Six 0·05 g. quantities of cholesterol were accurately weighed, and each intimately mixed with 25 g. soil. The mixtures were placed in separate porous flower pots in which the drainage holes had been closed with watch-glasses, and the pots buried with their tops 1 in. below the surface of a well-worked loamy garden soil. One group of three pots was watered weekly with rainwater, whilst the second group was treated with a dilute solution of $HgCl_2$.

Table 3. *Rate of disappearance of sterols from soil under different conditions*

Time months	Sterol content (mg.) Group I (water)	Group II ($HgCl_2$)
0*	50·0	50·0
3	42·7	49·2
6	29·6	49·8
12	18·3	48·9

* The sterol content of the untreated 25 g. sample of soil is negligible.

At intervals of 3, 6 and 12 months respectively, one pot from each group was removed, and the sterol content of the soil determined (Table 3). In the first group sterol disappeared, but the sterilizing action of the $HgCl_2$ inhibited microbiological activity in the second group so that the sterol content altered little. Similar experiments, using 'organic' cholesterol in the form of brain tissue and egg yolk, gave comparable results although disappearance of sterol was here less rapid than with the crystalline material.

The nature of the biological agents responsible for sterol breakdown is being investigated, and there are indications that decomposition is initiated by certain acid-tolerant, aerobic bacteria.

SUMMARY

1. A method is described for the determination of soil sterols.

2. The sterol content of a wide range of British soils has been determined.

3. Accumulation of sterols in soils occurs under conditions of extreme acidity, waterlogging and lack of aeration.

4. Where these conditions are reversed, as in arable soils, the sterol content gradually decreases.

5. The disappearance of soil sterols has been demonstrated experimentally, and has been shown to result from the activities of microbiological agents.

The author wishes to express his thanks to colleagues who have assisted in obtaining soil samples.

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Liver Catalase

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Stern [1935–6] isolated biliverdin (dehydrobilirubin) from the split products of horse liver catalase; the pigment was identified by Lemberg [cf. Lemberg & Wyndham, 1936–7]. Biliverdin was not present in the catalase preparation as such, but as a bile pigment haem compound bound to a protein similar to or identical with that characteristic of catalase [Lemberg & Wyndham, 1936–7]. After reduction

and transformation to pyridine-haemochromogen it gave the absorption spectrum of verdohaemochromogen. At that time it was assumed that the bile pigment haemoprotein was an impurity.

Sumner & Dounce [1937] obtained ox liver catalase in crystalline form. After repeated recrystallization fission with HCl in acetone of the enzyme yielded haemin and a blue compound, which was believed to contain iron and to be different from both biliverdin and verdohaemochromogen. While one-half of the catalase iron was firmly bound as protohaemin, the other half ('blue iron') was easily ionized by treatment with HCl, but unlike the iron of ferritin, which sometimes accompanies catalase, it was not precipitated with the protein. Iron of bile pigment haem compounds is readily set free by acid.

Lemberg, Norrie & Legge [1939] reported the isolation of crystalline biliverdin hydrochloride from crystalline ox and horse liver catalases. In view of the constant molar ratio of biliverdin to haemin (about 1 to 3), it was then assumed that the bile pigment haem formed an essential part of the catalase molecule. Another possibility, however, was mentioned, i.e. that the protohaem group in catalase may be oxidized to bile pigment haem by H_2O_2, leaving the protein component unaltered. At that time we were unable to find experimental support for this assumption.

Stern [1940] accepted this latter explanation of the presence of bile pigment haem in crystalline catalase as the correct one, while Agner [1939] and Theorell [1940] claimed that iron-free biliverdin was admixed with catalase. Agner based this claim on the isolation of a highly active horse liver catalase (of Kat.-f. 62,000),* which contained 0·086 % protohaem iron (83 % of the total iron in the form of protohaem and only 10 % in the form of 'blue iron'), but still gave biliverdin on splitting with acid. No quantitative estimations of the bile pigment were carried out, however, nor was any explanation given for the lack of success in separating catalase and biliverdin by fractional precipitation with $(NH_4)_2SO_4$, crystallization and cataphoresis.

Sumner, Dounce & Frampton [1940] obtained horse liver catalases in which the content of the iron accompanying biliverdin was inversely proportional to the specific activity. This indicated that the bile pigment haemoprotein is inactive and present in catalase in varying proportion.

We arrived at the same conclusions independently and with other methods. While Sumner studied the ionizable iron fraction, we investigated the molar ratio of bile pigment to haemin obtained by splitting catalase with acids, and the absorption spectra of the CO-haemochromogens obtained from catalase.

We have also shown that the bile pigment haem content of catalase can be increased by treatment with H_2O_2 in the presence of ascorbic acid, and that ascorbic acid inhibits catalase by accelerating its destruction by H_2O_2.

* Kat.-f. (Katalasefähigheit) was used by Euler to designate the activity of a solution containing 1 g. of catalase in 50 ml. We have previously used the translation 'catalase factor' in order to avoid the grammatical difficulties connected with the use of Kat.-f. A better translation is specific catalase activity (Sp.C.A.), and this will be used in this paper.

EXPERIMENTAL

Isolation and purification of catalase

Crystalline ox liver catalase was readily prepared by the method of Sumner & Dounce [1937]. The crystals can be further purified by washing with distilled water, and are dissolved by dialysing the suspension in distilled water against $M/50$ phosphate buffer pH 7·8; they are then recrystallized according to the method of Sumner & Dounce.

The crystals have the form of fine needles or small platelets described by Sumner, but on long standing in the refrigerator strong solutions of ox liver catalase occasionally deposit very large thick hexagonal plates which are no longer easily soluble in phosphate buffer.

Horse liver catalase. The dioxan method of Sumner *et al.* [1940] was not consistently successful in our hands, and we used the earlier stages of the method of Zeile & Hellström [1930]. The difficulty due to phosphate in the eluates [Agner, 1938] is easily overcome by removal of the phosphate by thorough dialysis at 0° against distilled water. After this the fractionation of catalase and ferritin even in strong solutions offers no difficulties. Crystallization and recrystallization were carried out according to Sumner *et al.* [1940]. Horse liver catalase crystals are more readily soluble in water than ox liver catalase.

Amorphous horse liver catalase was prepared by the methods of Zeile & Hellström [1930] and of Keilin & Hartree [1936].

Methods

Total protein was estimated with a micromodification of the method of Bick [1936].

Catalase activity. The activity was determined by the method of Zeile & Hellström [1930]. We can confirm that the values of $-(\Delta \log C_{H_2O_2})/\Delta t$ show a noticeable decrease with time even under their conditions. Sumner extrapolates these values for $t = 0$, but does not mention his method of extrapolation. By a different calculation one can determine the exact value of k_0.

According to Nosaka [1927] and Maximowitsch & Awtonomova [1928] the kinetics of destruction of H_2O_2 by blood catalase are given by the equations $-dC = k\,EC\,dt$ and $-dE = k'\,EC\,dt$, where C is the concentration of H_2O_2,

E that of catalase, k the reaction constant of the destruction of H_2O_2 by the enzyme and k' that of the destruction of the enzyme by H_2O_2. Integration yields the equation

$$\ln \frac{C_0}{C} - \ln \frac{C_0 + A}{C + A} = k' t A, \qquad (1)$$

where A is an integration constant defined as

$$A = \frac{k E_0}{k'} - C_0. \qquad (2)$$

If the H_2O_2 concentrations C_0, C_1, C_2 and C_3 at the times t_0, t_1, t_2 and t_3, where $t_3 - t_2$ equals $t_2 - t_1$, are known, kE_0, the required value for the activity at $t = 0$, can be calculated. The simplest way of finding A is by approximation from the equation

$$\ln \frac{C_1}{C_2} - \ln \frac{C_2}{C_3} = \ln \frac{C_1 + A}{C_2 + A} - \ln \frac{C_2 + A}{C_3 + A}. \qquad (3)$$

Inserting the value of A into equation (2) yields k' and from equation (1) kE_0 is then found as $(A + C_0)\,k'$. We have followed, however, the customary procedure of using $\log_{10}$ for calculation of the catalase reaction constant.

Table 1 shows that the course of H_2O_2 destruction by liver catalase can be satisfactorily calculated by assuming the validity of these equations, and that the action of liver catalase, therefore, obeys the same kinetic laws as that of blood catalase.

Table 1. *Kinetics of H_2O_2 destruction by liver catalase*

$A = 12$, $k' = 0{\cdot}0045$

t min.	$C_{H_2O_2}$ found as average of 2 estimations	$C_{H_2O_2}$ according to theory
0	9·03	9·03
0·5	8·11	8·07
1·5	6·62	6·64
3·5	4·59	4·55
4·5	3·86	3·85
6·5	2·81	2·75
7·5	2·41	2·35
9·5	1·80	1·75
10·5	1·56	1·53
20·0	0·43	0·45

In several instances we have observed a slight fall of A with time which would indicate that the speed of destruction of the enzyme does not decrease as much as the H_2O_2 concentration. The curvature of the graph of $C_{H_2O_2}$ plotted against time is slightly less than expected from theory.

A simpler calculation of k_0 can be derived from the equations: $-dC = kCE\,dt$ and $-dE = k'E$. It follows

$$\ln C_0 - \ln C = \frac{k}{k'} E_0 (1 - e^{-k't}). \qquad (4)$$

The destruction of the enzyme does, of course, depend on the H_2O_2 concentration, and the curvature of the $C_{H_2O_2}$ graph is greater than would be expected from equation (4). Under the conditions of the estimations, however, with a low enzyme concentration and a small fall of H_2O_2 concentration, the equation

$$k_0 = \text{antilog.} \left\{ \frac{\frac{1}{2}(t_1 + t_2) \log k_{t_1 - t_2} - \frac{1}{2}(t_2 + t_3) \log k_{t_2 - t_3}}{\frac{1}{2}(t_3 - t_1)} \right\}, \qquad (5)$$

which is derived from equation (4) yields results which agree with those found by the formulae (1)–(3) within the experimental error.

In order to compare our results with those of Sumner, we have calculated k_0 from the examples given by Sumner & Dounce [1939, p. 442] with our formulae. The values given by these authors are 0·0510 and, with half the enzyme concentration, 0·0247. With the formulae (1)–(3) we find 0·0511 and 0·0254, with formulae (5) 0·0505 and 0·0248. Our results are, therefore, strictly comparable with those of Sumner. Sumner & Dounce do not mention the volume of diluted catalase solution added to 50 ml. of H_2O_2, but if this was 0·5 ml., as used by Zeile, the specific activities were 24,000 and 23,200, and not 35,000 and 34,500 as given by them.

Protohaem in catalase was determined spectrophotometrically by measurement of the absorption at 558 mμ of the haemochromogen formed on adding NaOH, final conc. 1·25 %, followed by a few mg. $Na_2S_2O_4$. We have found no difference in the height of the absorption band on the addition of pyridine, nor any cloudiness of the solution, except with partly denatured catalase. The concentration of protohaemin in mg./ml. is $(\epsilon_{555\,m\mu} \times 10^3)/47{\cdot}5l$, where l is the length of layer through which the light passes, and 47·5 the extinction coefficient of a solution of protohaemochromogen containing 1 mg. haemin/ml.

Estimation of bile pigment content and bile pigment:haemin ratio. The isolation of bile pigments from catalase treated with acetic acid was by the method of Lemberg, Lockwood & Legge [1941*b*]. About 5 ml. of catalase solution of $k = 5000$ sufficed. No ascorbic acid was added, since acidified catalase does not oxidize biliverdin. The 66 % acetic acid solution was diluted with the same volume of peroxide-free ether, and the almost colourless protein removed by washing with Na acetate solution and water. These washings were saturated with $(NH_4)_2SO_4$, and re-extracted with ether [cf. Lemberg *et al.* 1941*b*, p. 369]. The bile pigments were extracted by repeated shaking with 20 % HCl.

The isolation of crystalline biliverdin hydrochloride from crystalline liver catalase has been described [Lemberg *et al.* 1939]. The crystals possessed the typical form and optical and solubility properties, and on oxidation of the complex zinc salt with iodine [cf. Lemberg, 1935] the characteristic fluorescence and absorption band of the biliviolin zinc complex salt were obtained.

In our first estimations we determined biliverdin in the acid extracts colorimetrically, by comparison with a biliverdin standard prepared from pure crystalline biliverdin. Later it was recognized that the extracts contain small amounts of biliviolin (usually about 20 % of total bile pigment). The values for bile pigment content and for the molar ratio (bile pigment Fe)/(bile pigment Fe + protohaem Fe), obtained by this method, are therefore too small, but can be compared among themselves. The results obtained by this method are given in Table 2.

In the later experiments, summarized in Table 3, biliverdin and bilivioIin were measured spectrophotometrically according to method III [Lemberg *et al.* 1941*b*].

After extraction of the bile pigments the ethereal solution of haemin was washed once with water, and protohaem was then extracted with NH_3 and determined spectrophotometrically as pyridine-haemochromogen [Lemberg, Legge & Lockwood, 1941*a*]. Usually 50–70 % of the total protohaem of catalase was thus recovered. The remainder clings to the denatured protein. We shall give below evidence showing that the bile pigment in catalase is present in the form of a bile pigment haem compound, from which bile pigment is split off quantitatively. The ratio (bile pigment Fe)/(bile pigment Fe+protohaem Fe), the latter being called 'total haem Fe', given in Tables 2 and 3, was calculated assuming that the recovery of bile pigment was as complete as that of protohaem.

Bile pigment and haemin yields from liver catalases

With the earlier method we found the ratio of bile pigment Fe to total haem Fe to be close to 0·25 for a number of preparations of ox and horse liver catalase of varying purity (Table 2). This suggested that catalase contained three protohaem groups and one bile pigment haem group in the molecule. With the more exact later method, however, a higher ratio was established, varying between 0·30 and 0·50 with an average of 0·38 (Table 3). This value does not suggest a stoichiometric ratio of bile pigment haem and protohaem, and the assumption of compounds between catalase and bile pigment haemoprotein, which contain one, two or three bile pigment haem groups in addition to three, two or one protohaem groups respectively, is thus not supported by experimental evidence.

Tables 2 and 3 show that the protohaem contents of the catalase preparations increase with their specific activities. Laskowski & Sumner [1941] have isolated crystalline ox erythrocyte catalase of the specific activity 48,000, and have found it to be free from bile pigment haem; the latter is, therefore, not necessary for catalase action. At first sight the experiments summarized in Tables 2 and 3 seem to support the view that the bile pigment haemoprotein is catalytically as active as the protohaem compound. The ratio of bile pigment Fe to total haem Fe does not vary with the specific activity over a wide range. A closer analysis reveals, however, that the bile pigment haemoprotein is inactive. The variations of the specific activity in our experiments are partly associated with factors other than that of the varying bile pigment content. The last column of Table 3 shows that the percentage of total haem Fe in catalase only occasionally approaches (e.g. preparations D 2*a*, D 2*c* and F 2 of ox liver catalase and D 1 of horse liver catalase) the theoretical value of 0·099 %, calculated for the presence of four haem groups in a catalase molecule of the molecular weight 225,000 [cf. Sumner *et al.* 1940]. The preparations with a lower total haem Fe content presumably still contain some inactive protein without haem groups, although some are crystalline. By multiplication with a correction factor (0·099 divided by percentage total haem Fe found) the true specific activity of the haemoproteins is obtained. If this is plotted against the ratio protohaem Fe/total haem Fe (Fig. 1), it is clearly seen that specific activity increases with decreasing bile pigment content. All but one of the points lie close to a straight line. This line cuts the 0-point, showing that the bile pigment haemoprotein is inactive, and reaches 52,000 for pure catalase without bile pigment haem. There is no significant difference between the activities of ox and horse liver catalases, and their variations with bile pigment haem content. The value 52,000 is close to that found by Sumner for the specific activity of ox erythrocyte catalase (48,000), which is free from bile pigment haem, and for the purest horse liver catalases.

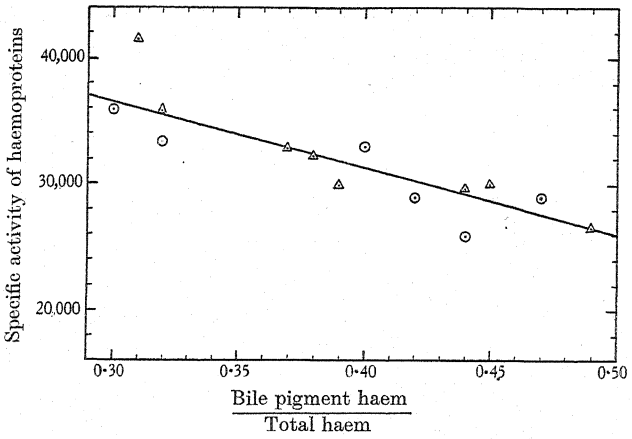

Fig. 1. Increase of specific activity with decreasing bile pigment haem content. △ Ox liver catalase. ⊙ Horse liver catalase.

Sumner *et al.* [1940] worked with a number of catalase preparations with more widely varying bile pigment haem contents, most of them of approximately equal purity, as the total haem Fe values (Table 2 of their paper) show. The specific activities, therefore, decrease with increasing bile pigment content without any correction for the presence of non-haematin compounds. It is more difficult to

Table 2. *Bile pigment and haemin yields from ox and horse liver catalases*

No.	Preparation		Specific activity	Protein conc. %	Haemin % of total protein	μg. per ml. Extracted proto-haem Fe	Bili-verdin	Bile pigment Fe	Ratio: bile pigment Fe / total haem Fe	*k*/proto-haem Fe
						Proto-haem Fe				
			Ox liver catalase							
K2	Zeile	0·1 %NH_3 eluate from Al_2O_3*	1,800	3·40	0·07	1·94	0·94	3·26	0·31	0·25	1,600
L1	,,	Crude extract	6,700	0·60	0·25	1·27	0·48	1·70	0·16	0·25	1,580
A2*b*	Sumner & Dounce	Eluted with NH_3 after A2*a*	10,700	0·90	0·22	1·70	0·84	3·10	0·30	0·26	2,640
C1	,,	Crystalline	—	—	—	—	9·32	33·5	3·21	0·25	—
A2	,,	,,	12,500	0·60	0·31	1·58	0·56	3·19	0·30	0·35	2,380
A2*a*	,,	A2 adsorbed to Al_2O_3 and re-eluted	—	—	—	1·57	0·81	3·81	0·36	0·31	3,120
A1	,,	Crystalline	17,500	1·90	0·31	5·06	3·25	13·0	1·25	0·27	3,260
B2	,,	Mother liquor of B1	17,000	0·90	0·33	2·57	1·67	4·7	0·45	0·21	2,960
B1	,,	Crystalline	—	—	—	—	8·45	36·4	3·48	0·29	—
B1′	,,	B1 recrystallized	33,000	1·20	0·73	7·53	4·28	14·4	1·38	0·24	2,630
			Horse liver catalase								
A1	Zeile	Crude extract	5,000	4·6	0·26	10·1	1·68†	4·6	0·44	0·21	1,140
A2	,,	After adsorption to Al_2O_3	25,700	3·82	0·50	19·2	12·3	46·4	4·43	0·27	2,560
B1	Keilin	Amorphous	20,000	4·28	0·48	20·7	9·9	44·0	4·20	0·28	2,080
B2*a*	,,	1st fraction of ammonium sulphate ppt.	—	—	—	20·8	11·6	33·6	3·21	0·22	—
B2*b*	,,	2nd fraction	—	—	—	17·0	9·0	32·4	3·10	0·25	—
C2	Zeile, Agner, Sumner	Crystalline	—	—	—	8·75	4·93	16·0	1·53	0·24	—

* Eluate after main part of catalase had been eluted with secondary phosphate.
† The small percentage of haemin recovery after splitting makes the result uncertain.

Table 3. *Bile pigment and haemin yields from ox and horse liver catalases*

No.	Preparation		Specific activity	Protein conc. %	Haemin % of total protein	μg. per ml. Extracted proto-haem Fe	Proto-haem Fe	Bili-verdin	Bili-violin	Bile pigment Fe	Ratio: bile pigment Fe / total haem Fe	*k*/proto-haem Fe	Total haem Fe % of total protein
						Ox liver catalase							
H2	Sumner & Dounce	Inactivated extract*	2,500	2·07	0·66	11·75	4·70	14·7	20·9	3·40	0·42	220	0·098
H1	,,	Crystalline	—	—	—	1·18	0·33†	2·30	0·88	0·30	0·45	2,920	0·050
E2	,,	2nd fraction of crystals	15,000	1·60	0·36	4·96	2·16	12·3	1·5	1·32	0·38	2,510	0·050
E3	,,	Amorphous	19,700	0·62	0·43	2·28	0·81†	5·40	1·33	0·64	0·44	2,680	0·066
D1	,,	Amorphous	21,800	0·78	0·50	3·29	2·00	11·2	0·95	1·16	0·37	2,580	0·067
J1	,,	Crystalline	24,600	0·50	—	—	1·36	4·06	0·91	0·48	0·26	—	—
D2*c*	,,	,,	24,600	0·88	0·54	4·08	2·36	7·03	4·37	1·09	0·32	2,650	0·068
D2*b*	,,	,,	25,700	1·44	0·55	6·76	1·98†	15·0	6·9	1·94	0·49‡	2,740	0·093
E1	,,	,,	26,200	0·82	0·59	4·16	2·04	8·6	3·3	1·14	0·36	2,580	0·079
D2*a*	,,	,,	27,300	0·20	0·58	0·99	0·61	4·15	1·03	0·50	0·45	2,770	0·090
F2	,,	,,	36,400	0·90	0·70	5·39	3·45	11·9	4·3	1·55	0·31	3,040	0·087
						Horse liver catalase							
M1	Zeile, Agner, Sumner	Crystals	—	—	—	—	1·45	6·9	1·0	0·75	0·34	—	—
C2	,,	Mother liquor of crystals	—	—	—	—	3·42	28·8	6·2	3·35	0·49	—	—
G	,,	Crystalline	13,800	0·92	0·31	2·45	1·58	10·3	1·9	1·15	0·42	2,500	0·047
H	,,	,,	19,000	1·29	0·47	5·20	2·80	17·0	6·5	2·24	0·44	2,350	0·073
FI*b*	,,	,,	18,400	0·84	—	—	2·49	13·6	2·4	1·53	0·38	—	—
F2	,,	Amorphous	23,200	1·01	0·51	4·41	2·20	8·8	1·65	1·00	0·31	2,580	0·064
FI	,,	,,	24,000	1·95	0·51	8·53	4·22	24·0	4·2	2·69	0·39	2,750	0·072
FI*a*	,,	Crystalline	24,000	1·80	0·57	8·68	5·42	18·9	7·8	2·56	0·32	2,350	0·071
D1	,,	,,	28,000	1·32	0·60	6·75	1·84†	16·6	0·4	1·62	0·47	2,730	0·091
FII*a*	,,	,,	30,600	0·72	—	—	2·72	11·8	0·7	1·19	0·30	—	—
FII*b*	,,	,,	31,200	0·43	—	—	1·57	5·09	2·71	0·74	0·32	—	—

* After long standing of the crude extract; remarkably much inactive haematin and biliviolin.
† The small percentage of haemin recovery after splitting makes the result less certain.
‡ According to the absorption spectrum of the CO-haemochromogen: 0·38.

Table 6. *Increase of bile pigment haem in catalase by treatment with H_2O_2 in the presence of ascorbic acid*

Liver catalase: Species	Vol. ml.	*k*	Ascorbic acid mg. per 100 ml.	*t*°	H_2O_2 g.	mg. H_2O_2 / mg. active enzyme	Bile pigment Fe / Total haem Fe: Before oxidation	After oxidation	Total activity decrease %	Activity decrease due to oxidation of prosthetic group %	*dC*/*dE* mg. H_2O_2 destroyed / mg. enzyme inactivated	Specific activity: Before oxidation	After oxidation	Decrease %	*k*/protohaem Fe: Before oxidation	After oxidation
Ox	15	6,150	5	30	62·5	1,700	0·26	0·58	83	44	2,000	24,600	18,300	25	—	—
Horse	10	12,200	5	30	45	900	0·44	0·49	65	9	1,400	—	—	—	2,350	1,650
Horse	10	4,530	5	30	21	1,200	0·32	0·52	49	30	2,350	27,000	23,100	15	2,590	1,850
Horse	22	6,340	25	5	66	1,200	0·42	0·48	34	10	3,300	13,800	13,000	6	—	—
Horse	22·2	6,340	30	23	67·5	1,200	0·42	0·46	19	7	6,500	—	—	—	2,500	2,400
Horse	20	6,340	40	37	30	600	0·42	0·43	74	2	800	—	—	—	2,500	2,090

draw a straight line through the points of Sumner's table in a diagram similar to Fig. 1, with or without correction for presence of non-haematin compounds, and the line does not appear to go through the 0-point. This is probably owing to the way in which the bile pigment haem Fe is determined by Sumner as 'blue iron' fraction. Even the best catalase preparations still contain small amounts of ferritin, a protein with high iron content, and it is likely that a small part of the ferritin iron is not precipitated with the protein, but is determined as 'blue iron'. Our method takes into consideration only haem and bile pigment haem, and the presence of iron in any other form, e.g. as ferritin, does not interfere with the results. Apart from this minor difference, however, our results independently confirm those of Sumner by different methods.

Spectrophotometric study of the catalase haemochromogen

While Zeile [1933] claimed that the haemochromogen prepared from catalase is identical with that from protohaem, Lemberg & Wyndham [1936–7] observed an absorption band at 651 mμ with the pyridine haemochromogen prepared from highly active, but amorphous horse liver catalase.

The same band is observed, when crystalline preparations of ox and horse liver catalase are studied. NaOH, final conc. 1 %, is added to catalase and after the addition of a few mg. $Na_2S_2O_4$ the absorption spectrum is immediately observed. With the spectrophotometer the ratio $\epsilon_{650} : \epsilon_{557}$ is found to be 0·25, while for protohaemochromogen, prepared in the same manner from haemoglobin, this ratio is only 0·015. This absorption in the red part of the spectrum is not due to unspecific background absorption, as Stern [1937] assumed, since a definite maximum of absorption at 650 mμ is found. It cannot be due to biliverdin either, since biliverdin is reduced by $Na_2S_2O_4$ in alkaline solution to a yellow compound which does not absorb in the red.

The presence of another haemochromogen in addition to protohaemochromogen is confirmed by examination of the CO-haemochromogen. By bubbling coal gas through the solution, previously saturated with the gas, during the addition of $Na_2S_2O_4$ and NaOH, the danger of secondary oxidation by H_2O_2 formed from $Na_2S_2O_4$ is prevented. A strong absorption band at 630 mμ is found, which is usually about half as high as that of CO-protohaemochromogen at 570 mμ (Fig. 2).

The absorption curve differs considerably from that of CO-protohaemochromogen prepared from haemoglobin.

While the haemochromogen accompanying protohaemochromogen is similar to cholehaemochromogen in some properties, differences exist between them. The position of the absorption band of the CO-compounds is the same, and removal of CO causes the same shift to 618 mμ; the absorption band at 630 mμ of the catalase compound is, however, accompanied by absorption rising towards the infra-red. A further difference is that cholegloblin gives only a poor yield of biliverdin, while bile pigment formation from the catalase haemoprotein appears to be quantitative. While such differences

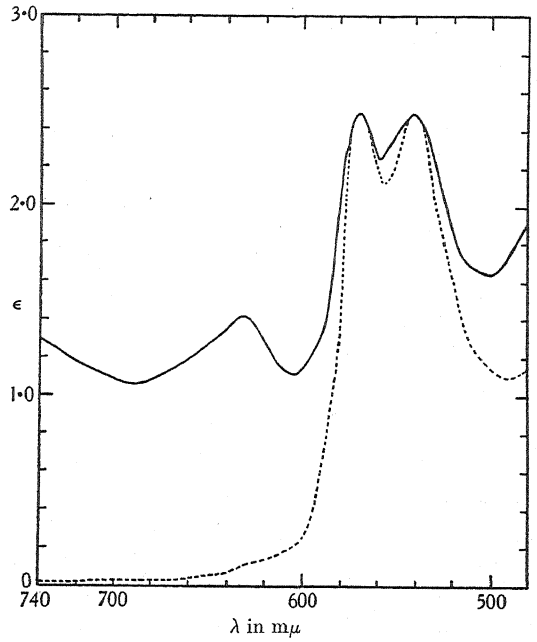

Fig. 2. CO-haemochromogen absorption curve. —— CO-haemochromogen from catalase. ---- CO-haemochromogen from haemoglobin.

may be due to the different proteins to which the bile pigment haem group is linked, the fact that the absorption band of the haemochromogen before the action of CO is found at 650 mμ, not at 618 mμ, suggests a prosthetic group in catalase slightly different from cholehaem. It seems to constitute a link between verdohaem and cholehaem.

The bile pigment haemochromogens have been found to possess approximately equal molar extinction coefficients at the respective maxima of absorption. We may therefore assume that the CO-haemochromogen from catalase has the same molar extinction at 630 mμ as CO-cholehaemochromogen, and can then calculate the ratio of bile pigment haem to total haem as

$$(\epsilon_{630}/\epsilon_{570} - 0{\cdot}04)/(0{\cdot}55\epsilon_{630}/\epsilon_{570} + 0{\cdot}96).$$

This formula is in principle the same as that given by Lemberg *et al.* [1941*a*], except that it gives the ratio bile pigment haem/total haem instead of bile pigment haem/protohaem as in the earlier formula.

Table 4 shows that the ratio found in this way is the same as that found by determinations of bile pigment and haemin in the split products. With one exception (D 2*b*) the results agree very well. It can

Table 4

Ox liver catalase preparation	Molar ratio: bile pigment haem / total haem — Found by estimation of the split products	Found by spectrophotometric analysis
F 2	0·31	0·35
D 2*c*	0·32	0·33
E 1	0·36	0·38
D 1	0·37	0·40
E 2	0·38	0·38
L	0·40	0·42
E 3	0·44	0·44
H 1	0·45	0·43
D 2*a*	0·45	0·43
D 2*b*	0·49	0·38

be concluded from these experiments that the biliverdin obtained from catalase by splitting with acids, occurs in catalase in the form of a bile pigment haemoprotein.

OXIDATION OF CATALASE TO BILE PIGMENT HAEMOPROTEIN BY H_2O_2

Calculation of the minimum amount of H_2O_2 required. We have reported [Lemberg *et al.* 1939] that we could not observe an increase in the bile pigment haem content of catalase when we allowed the enzyme to destroy gradually a large amount of H_2O_2.

From the kinetics of the enzyme reaction given above we can now calculate how much H_2O_2 catalase can destroy before it is inactivated, and thus determine the minimum amount of H_2O_2 with which the enzyme must react in order to obtain an experimentally verifiable increase in its bile pigment haem content. These calculations show that the amounts of H_2O_2 required are so large that the experimental difficulties are insurmountable, even if the inactivation of catalase in dilute solution were due entirely to the oxidation of protohaem to bile pigment haem.

From the two equations

$$-dC = kEC\,dt \quad \text{and} \quad -dE = k'EC\,dt$$

it follows that

$$\frac{dC}{dE} = \frac{k}{k'} = \frac{A + C_0}{E_0},$$

where $A + C_0$ is measured in terms of ml. $KMnO_4$/10 ml. H_2O_2 solution. C_0 ml. $KMnO_4$ are equivalent to 10 ml. $N/100$ H_2O_2 and $A + C_0$ therefore to $(A + C_0)/100C_0$ mol./l. E_0 in mol./l. is determined as $20k_0/225{,}000K$, where k_0 is the initial velocity constant of H_2O_2 destruction, and K the specific activity. Since we are interested only in the oxidation of the active catalase molecules, the maximum specific activity (50,000) is used. Thus we have:

$$\frac{dC}{dE} = 5{\cdot}62 \cdot 10^6 \frac{A + C_0}{C_0 k_0} \frac{\text{g. mol. } H_2O_2 \text{ destroyed}}{\text{g. mol. catalase inactivated}}$$

$$= 850 \frac{A + C_0}{C_0 k_0} \frac{\text{g. } H_2O_2 \text{ destroyed}}{\text{g. enzyme inactivated}}$$

or $$850 \frac{1}{C_0 k'} \frac{\text{g. } H_2O_2 \text{ destroyed}}{\text{g. enzyme inactivated}}.$$

k' was not determined with great accuracy; at 0° it varied from 0·0007 to 0·0023 with an average of 0·0014. No significant difference was found between the k' of ox and of horse liver catalase. dC/dE varies from 20,000 to 75,000 g. H_2O_2/g. catalase, with an average of 35,000.

In order to inactivate 10 mg. out of 50 mg. active enzyme (the minimum needed for estimation) more than 1100 ml. of 30 % H_2O_2 solution would have to be added. This would increase the ratio of bile pigment Fe to total haem Fe from 0·40 to 0·52, provided that the inactivation of the enzyme were entirely due to the oxidation of haematin to bile pigment haematin.

Effect of ascorbic acid on the destruction of catalase by H_2O_2. We have found, however, a way to overcome these difficulties. Various authors have observed that the action of catalase is inhibited by reducing substances, e.g. glutathione and ascorbic acid. Marks [1936] found that ascorbic acid and reduced glutathione increase the rate of inactivation of catalase by atmospheric O_2. By the action of ascorbic acid and H_2O_2 haemoglobin and haemochromogens are oxidized to bile pigment haem compounds. We have therefore investigated whether the inhibition of the enzyme action by ascorbic acid is due to a real diminution of k, or to a faster destruction of the enzyme by H_2O_2 (increase of k').

Ascorbic acid reacts only slowly with dilute H_2O_2 or $KMnO_4$. If the first drop of $N/100$ $KMnO_4$ solution giving a colour lasting for more than 10 sec. is taken as the end-point, the presence of ascorbic acid in a concentration not greater than 10 mg./100 ml. does not interfere with the titration. Moreover there is no measurable reaction between H_2O_2 and ascorbic acid during 10 min.

Table 5 shows that k_0 is decreased little, if at all, by ascorbic acid, while k' is greatly increased. Ascorbic acid inhibits the enzyme action by accelerating the destruction of catalase by H_2O_2. The ratio dC/dE is thus diminished from 35000 to 4000 by a concentration of 10 mg. ascorbic acid/100 ml.

Experiments with glutathione failed to yield clear-cut results. Reduced glutathione reacts too rapidly with H_2O_2, and also with $KMnO_4$. Nevertheless our experiments indicate that the inhibition of catalase activity by reduced glutathione is not due to increased destruction of the enzyme.

Table 5. *Destruction of catalase by H_2O_2 at 0° in the presence and absence of ascorbic acid*

	k_0	k'	$\frac{dC/dE}{\text{g. } H_2O_2 \text{ destroyed}}{\text{g. catalase inactivated}}$
Horse liver catalase without ascorbic acid	0·054	0·0018	29,000
Horse liver catalase with 2 mg./100 g. ascorbic acid	0·054	0·0074	7,100
Horse liver catalase with 10 mg./100 g. ascorbic acid	0·054	0·0127	4,100
Ox liver catalase without ascorbic acid	0·056	0·0017	27,000
Ox liver catalase with 4 mg./100 g. ascorbic acid	0·052	0·0088	4,700

Large-scale experiments. These were made to see if treatment of catalase with H_2O_2 in the presence of ascorbic acid increases the bile pigment haem content. Much higher concentrations of catalase and, at least locally, of H_2O_2 cannot be avoided in these experiments. Otherwise the large dilution of the enzyme would make impossible its recovery in the concentrated form required for bile pigment estimations. Even the use of concentrated (30 %) H_2O_2 causes much dilution. Concentrated H_2O_2, however, destroys the enzyme by denaturing the protein. The danger of this was minimized by gradual addition and thorough mixing.

30 % H_2O_2 (Schering Kahlbaum *pro analysi*) was neutralized with NaOH and slowly dropped from a micro-burette, at a rate of one drop every 7–10 sec., into the dilute well-buffered (*p*H 6·8) catalase solution containing the ascorbic acid. The mixture was kept in a continuous swirling motion, and the drops of H_2O_2 were not allowed to fall into the froth; gentle swirling by hand was best. Occasionally octyl alcohol had to be added, but this caused some enzyme denaturation and was avoided if possible. It can be calculated that with the enzyme concentrations used all the H_2O_2 was almost completely destroyed before the next drop was added.

In the preliminary experiments, to 0·5 ml. of a catalase solution ($k = 7000$) 5 ml. of 30 % H_2O_2 were added as described; temp. $< 5°$. In a second experiment ascorbic acid was added (5 mg./100 ml.). Without ascorbic acid 26 % of the enzymic activity was destroyed ($dC/dE = 4200$), with ascorbic acid 59 % ($dC/dE = 1800$). Ascorbic acid thus increased the destruction of catalase by H_2O_2 under these conditions, but not as much as in the experiments in very dilute solutions (cf. Table 5). This suggests that in the large-scale experiments much catalase is destroyed in a different way, a way which is much less or not at all accelerated by ascorbic acid. If it is assumed that the enzyme destruction in dilute solution in the presence of ascorbic acid is entirely due to the oxidation of protohaem to bile pigment haem and this reaction itself remained unaltered in the large-scale experiments, it can be calculated that the destruction of 60 % of the activity of the enzyme in the experiment above ought to be accompanied by a significant increase in the bile pigment haem content. dC/dE in the large-scale experiment (1800) is about one-third of dC/dE in dilute solution (4700, cf. Table 5), i.e. three times as much enzyme is destroyed by the same amount of H_2O_2 in the large-scale experiments as in dilute solution, in which we assume the destruction to be due to the oxidation of protohaem to bile pigment haem. If, of the 60 % total loss of activity, 20 % is due to oxidation of protohaem to bile pigment haem, an original ratio of bile pigment haem to total haem of 0·40 ought to be increased to 0·52, which is far beyond the range of experimental error. The large-scale experiments were carried out at temperatures from 5 to 37°. Since the inactivation of the enzyme by H_2O_2 has a higher temperature coefficient than the catalytic destruction of H_2O_2, higher temperatures ought to be favourable, up to the critical zone, where temperature inactivation of the enzyme becomes significant. After the addition of H_2O_2 the mixtures were worked up in the following way. The catalase activity was determined as usual. For the other estimations the catalase was concentrated by precipitation with $(NH_4)_2SO_4$. In spite of being finely dispersed the precipitate was easily collected on a Whatman no. 42 filter; the water-clear filtrate was practically inactive. The catalase was dissolved in secondary phosphate solution and dialysed at 0° in a collodion bag.

The experiments summarized in Table 6 (p. 122) show that in five of six experiments a definitely higher bile pigment haem content was found after treatment with H_2O_2. In the one experiment in which no significant increase was found, H_2O_2 had been added more rapidly at 37°, and the fact that a smaller amount of H_2O_2 had led to the same degree of enzyme destruction (see columns 5 and 8), indicates greater denaturation of protein.

The quotient dC/dE and that part of enzyme inactivation due to the oxidation of protohaem to bile pigment haem vary considerably. This is not surprising in view of the experimental difficulties. The degree of denaturation of catalase by conc. H_2O_2 may alter according to froth formation, addition of octyl alcohol and efficiency of mixing. The ascorbic acid concentration is altered during the addition of H_2O_2 by dilution and by oxidation, and while the former was avoided by adding ascorbic acid from time to time, the degree of the latter was unknown.

We had expected that these experiments would supply further evidence that the bile pigment

haemoprotein is catalytically inactive, but no conclusive evidence was obtained. The determination of protohaem as haemochromogen is more difficult than with untreated catalase and is disturbed by cloudiness of the solution and incomplete reduction by $Na_2S_2O_4$. If the values for protohaem are correct, H_2O_2 causes a fall of the quotient k/protohaem Fe, i.e. some inactivation of catalase without precipitation or oxidation, and the picture is further complicated by the fact that the decrease of the specific activities of the recovered solutions is smaller than would correspond to the decrease of total activity. The latter must be explained by the denaturation and removal of inactive proteins in subsequent treatment.

No difficulties were, however, encountered in the determination of protohaem after acid splitting and of the ratio bile pigment Fe to total haem Fe. Duplicate estimations of this ratio gave results within $\pm 0{\cdot}02$ and usually $\pm 0{\cdot}01$. With the exception of the experiment in the last row of Table 6, which may be considered as a blank, the increases of the bile pigment haem content are significant.

DISCUSSION

In earlier experiments we have found that by the action of H_2O_2 in the presence of ascorbic acid the protohaem group of haemoglobin and of haemochromogens is oxidized to bile pigment haem. Analogous reactions with a slightly modified mechanism (coupled oxidation), but of principally the same type, occur *in vivo*. They underlie the physiological formation of bile pigment from haemoglobin as well as the intracorpuscular biliverdin formation by phenylhydrazine [Lemberg & Legge, 1942] and other drugs. Ox and horse liver catalases contain bile pigment haem, while ox erythrocyte catalase has been found by Laskowski & Sumner [1941] to contain protohaem alone. Does catalase undergo an oxidation *in vivo* similar to haemoglobin?

In the present paper we have shown that by the action of H_2O_2 in the presence of ascorbic acid the protohaem groups of catalase are oxidized to bile pigment haem groups. These experiments had to be carried out with unphysiologically high concentrations of enzyme and H_2O_2 but we have shown the effect of ascorbic acid on the rate of the enzyme destruction in low concentrations, and it is likely that the mechanism is the same.

The bile pigment haem content of the liver catalase can be explained in three ways: (1) it is an artefact and due to the oxidation of catalase in post-mortem changes or during its isolation from the liver; (2) bile pigment haem groups formed by the breakdown of haemoglobin in the liver are incorporated in the synthesis of catalase; or (3) catalase itself undergoes an oxidation in the liver.

With regard to the first possibility, it might be argued that the presence of larger amounts of ascorbic acid in the liver than in the red cells may explain why an oxidation does not occur during the isolation of catalase from erythrocytes. By autoxidation of ascorbic acid H_2O_2 is formed and may act together with ascorbic acid on liver catalase which was originally free from bile pigment haem. The amounts of H_2O_2 which can be formed during the isolation cannot, however, be large and appear to be insufficient to explain an oxidation of 30–50 % of the prosthetic group. Even at 20° the inactivation of catalase by ascorbic acid is slow [Marks, 1936] and the isolation is carried out at 0°. Neither Sumner *et al.* [1940] nor we ourselves have found evidence supporting this assumption.

The second explanation does not appear probable if it is remembered how specific is the synthesis of haemoglobin. Porphyrins with side chains differing from those of protoporphyrin are not united with iron and globin, even though such compounds may still carry O_2, while if the catalase haemoprotein were synthesized from bile pigment haem and catalase protein, it would be inactive.

This leaves the third hypothesis, an *in vivo* oxidation of catalase, as the most likely one. The difference between erythrocyte and liver catalases would be explained by the greater content of reducing substances in the liver cells and their greater respiration.

If this is the correct explanation, it leads to important implications. Catalase can then no longer be considered as a mere safety valve against occasional H_2O_2 formation. H_2O_2 must be destroyed by the enzyme *in vivo* on a scale which is not negligible. Even in the presence of ascorbic acid in concentrations found in the liver cells, each molecule of catalase must have destroyed at least 2×10^7 molecules of H_2O_2 before it became converted into bile pigment haemoprotein. 1000 g. of horse liver contain 0·5–1·0 g. of active protohaem catalase according to the activity of the extracts [Stern, 1935–6], which would mean the destruction of at least 500–1000 g. of H_2O_2 by 1000 g. of liver during the lifetime of the enzyme. We have not yet any definite data on the latter, but to judge from the fact that the liver catalase of a colt did not contain less bile pigment haem than that of the adult horse [Sumner *et al.* 1940] it is probably to be measured in weeks or months rather than in years.

The method of determining the bile pigment content of catalase may be used for studying the metabolism of catalase, but since large amounts of catalase are required for the estimations, other methods, e.g. with tracer elements, would be preferable.

Finally our experiments lend support to the findings of Keilin & Hartree [1938] that ferric

catalase iron is reduced to ferrous iron during its action on H_2O_2. Only ferrous haem compounds are oxidized by H_2O_2 to bile pigment haem compounds. Ascorbic acid does not reduce catalase, but may protect the ferrous H_2O_2-catalase compound against autoxidation.

SUMMARY

1. A simplified procedure for the isolation of crystalline horse liver catalase is described.

2. By splitting with acetic acid and extracting with ether, biliverdin and small amounts of biliviolin are obtained from crystalline ox and horse liver catalases.

3. The molar ratio of (bile pigment)/(bile pigment + haem) from impure and pure horse and ox liver catalases was found to vary from 0·3 to 0·5.

4. The CO-haemochromogens of these catalase preparations had, in addition to the absorption bands in CO-protohaemochromogen, an absorption band in the same position as that of CO-cholehaemochromogen. Spectrophotometric analysis showed that the height of this band was proportional to the bile pigment content as found by the splitting with acids, and that the same values for the ratio of (bile pigment Fe)/(bile pigment Fe + haem Fe) were found by both methods. This shows that the bile pigments are derived from a bile pigment haemoprotein.

5. If the presence of inactive haem-free protein in the impure preparations is taken into consideration, it can be shown that the specific activity (Kat.-f.) increases with decreasing bile pigment haem content, being about 50,000 for catalase containing protohaem only, and nil for the bile pigment haemoprotein.

6. A theoretically sound method for measuring catalase activity is described, which gives also the rate of destruction of the enzyme by H_2O_2.

7. Ascorbic acid inhibits catalase by accelerating the destruction of the enzyme by H_2O_2.

8. By the action of H_2O_2 on catalase in the presence of ascorbic acid, bile pigment haemoprotein is formed from protohaem catalase.

9. The significance of these findings for the explanation of the bile pigment haem content in liver catalase is discussed.

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The Technique of the Biological Vitamin A Assay

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The publications of Burn [1937], Irwin [1937], Coward [1938] and Bliss & Marks [1939], to cite a few of the more important papers, testify to the existence of a good measure of agreement on the general principles of the graded-response biological assay. At the same time assays of any particular substance usually necessitate the use of some *ad hoc* technique. The rat-growth (curative) vitamin A

assay is a notable example, and we here outline recent developments in the conduct of this assay in our laboratory.

Some special considerations regarding the vitamin A assay are listed below:

(i) The criterion of vitamin A depletion in the experimental animals is cessation, or marked retardation, of growth, a condition liable to cause secondary reactions that may jeopardize the response to vitamin A.

(ii) The useful range of the growth-response curve is limited at its lower end by an abrupt fall at the point where the dose is just insufficient to prevent the onset of a pathological state, with consequent invalidity of response, i.e. the curve does not rise smoothly from zero.

(iii) If the behaviour of the rats during the depletion period is 'good' there will be occasional cases of death or loss of weight. There is no biometrically acceptable method of incorporating the records of such animals into the assay: if the rats die during the test period there is clearly no alternative to the somewhat unsatisfactory procedure of, in effect, ignoring the existence of the behaviour records; and because of this, the records of rats that lose weight have also, *a fortiori*, to be set aside. Otherwise we should have the paradox that while a negative growth pulled down the group average, loss of weight culminating in early death left the average undisturbed. It is therefore desirable that the assay conditions be such that there is a maximum number of positive growth responses at all dosage levels.

(iv) The International Standard is not vitamin A but the provitamin β-carotene. Some assurance that the two substances give a common kind of response under all conditions of the assay is therefore desirable, i.e. evidence of the constancy of the conversion of β-carotene to vitamin A is required. There is therefore need to demonstrate a substantial identity between the normal variations of response, and between the constants of slope of the curves of response, to Standard and test materials.

The inaugural work on our assay technique has been described [Morgan, 1934]. Minor changes have since been introduced, e.g. the vitamin A-deficient diet now in use is:

	%
Casein A/E	10
Coconut-cake meal	30
Flaked rice	48
Dried yeast	8
Steenbock's no. 40 salt mixture	4
	100

The addition of vitamin D has been shown to be unnecessary with this diet. The animals (bucks only) become depleted of vitamin A at the age of about 50 days, litters always *en bloc*, so that the members of each litter are started on the 3-week test on the same day. Typical average behaviour on the diet is portrayed in simplified form in Fig. 1.

Morgan [1934] found that for the then existing regime the logarithm of the dosage was linearly related to the square of the growth response. In practice the differences between the squares of

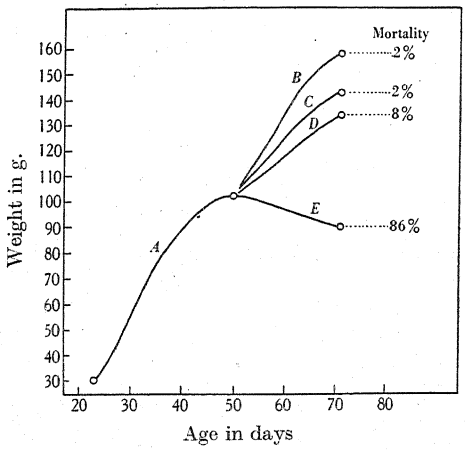

Fig. 1. Composite growth curves of 834 recent test rats. Curve A. From weaning to vitamin-A-depletion point. Curve B. Test period; positive controls; on $\leqslant 20$ i.u./day. Curve C. Test period; on 2 i.u./day. Curve D. Test period; on 1·2 i.u./day. Curve E. Test period; negative controls; no vitamin A.

response in weight-matched, isogenic, Standard (T): test substance (S) pairs were taken as metameters* with which to calculate directly $\sigma_{(S-T)}$ and to evaluate the 'dose ratio' (S/T), and hence the test-material potency, by substitution in

$$G_S^2 - G_T^2 = b \log S/T.$$

Until recently we have continued to use this equation. At approximately 6-monthly intervals the accumulated test data, covering a dosage range of about 0·5–3·0 i.u./day, were summarized to check the validity of the equation and to recalculate b. There is, of course, no *a priori* reason why the growth: log-dose curve should be linear in the vitamin A, or in any other, biological assay; the important consideration is whether accuracy is enhanced by the employment of a precise equation rather than the convenient linear approximation.

One of the consequences of the above technique was that the necessity of pairing left an unusable residuum of 10–15 % of the animals. This wastefulness caused us to investigate the rationale of rat selection; at the same time the biometry of the assay was reviewed, special attention being given to the stability of equation constants.

* Metameter = the measurement used in a biological assay (see *Biochem. J.* 1942, **36**, 411, footnote).

To investigate the pairing technique, the records of six routine assays were analysed. In anticipation of this work the usual strictness of weight matching ($\gtrless \pm 4$ g.) of the isogenic pairs had been relaxed. From each set of records two correlation coefficients were calculated, first, that within the paired Standard:test-material growth-responses, and secondly, that between initial-weight differences and growth-response differences in the individual pairs —after segregation of interlitter and interdose differences (see Table 1).

Table 1. *Correlations (a) within isogenically paired comparisons of growth-response and (b) between initial-weight differences and subsequent growth-response differences in the same pairs*

Assay no.	No. of (buck) pairs	(a) Correlation coefficient r_a	(b) Correlation coefficient r_b
i	27	+0·497	+0·024
ii	24	+0·302	+0·201
iii	21	+0·387	+0·118
iv	23	+0·474	−0·404
v	32	+0·478	+0·195
vi	20	+0·293	−0·010
Means (by z transformation)		+0·418	+0·030

The mean r_a is highly significant and indicates that the pairing technique is advantageous, while the mean r_b is not significant and indicates that weight-matching is superfluous [cf. Bacharach, 1940]. Thus isogenic pairing, or, in general, intralitter comparison, is the important factor. A corollary (obtained from the weighed means of the variance ratios) is that 10 pairs of isogenically matched rats give results comparable in accuracy with those of 19·3 arbitrarily selected pairs.

We looked, therefore, for a new assay plan. We observed that if a group of test animals is equally divided between four dosages, two levels of Standard and two of test material, the use of the 'internally' calculated best linear equation (growth:log-dose) automatically compensates for real deviations from the assumed relationship. To illustrate: suppose that the true relation between dosage (D) and growth response (G) obtaining in a group of rats used in an assay were expressible as the polynomial

$$G = 27 + 88 \log D - 60 (\log D)^2$$

(the formal expression of a typical 'square of the growth' equation such as we encounter), and that in the assay there were equal numbers of animals on 1 unit and 2 units Standard (S) and nominal 1 unit and 2 units test-material (T); ideally we can imagine the exclusive use of isogenic tetrads of bucks for this four-term dosage distribution. Suppose further that the true dose ratio T/S was 1·4 so that with a sufficiently large number of animals the growth responses would approximate to the following (calculated from the polynomial):

1 unit S : 27·00 g.
2 units S : 48·05 g.
Nominal 1 unit $T \equiv 1{\cdot}4$ units S : 38·58 g.
Nominal 2 units $T \equiv 2{\cdot}8$ units S : 54·35 g.
All doses 'daily'.

Let us now ignore the true growth-dosage equation and interpret the assay from the best internal linear equation. We have

Slope b, from Standard growths $= 21{\cdot}05/\log 2 = 69{\cdot}9$
Slope b, from test-material growths $= 15{\cdot}77/\log 2 = 52{\cdot}4$

The two values are, of course, significantly different. If, however, they are averaged and the mean (61·2) is used to evaluate the (supposedly unknown) dose ratio from the linear equation, we have

$$\text{Dose ratio} = \text{antilog} \frac{\frac{1}{2}(38{\cdot}58 - 27{\cdot}00) + \frac{1}{2}(54{\cdot}35 - 48{\cdot}05)}{61{\cdot}2} = 1{\cdot}40.$$

This compensatory effect operates over fairly wide limits: it certainly covers all curves in our experience. An incidental inference is that a statistical demonstration of departure from parallelism in a biological assay is not necessarily evidence of the invalidity of the test.

When the Standard : test-material dose ratio is unity the equation constants are of minimum importance in assay interpretation, so that the smaller the dose ratio the better the assay. Consequently we always repeat assays in which the dose ratio is found to be unexpectedly high or low; in the repetition appropriately readjusted test-material doses are used.

Because isogenic tetrads comprise only a few of the available test animals, others must also be used for routine work. The following scheme utilizes all rats to advantage:

Dosage levels:
1·2 and 2·0 units of Standard (S)
'nominal' 1·2 and 2·0 units of test-material (T)

Distributions:
In a four-buck litter: $S_{1\cdot2}$, $S_{2\cdot0}$, $T_{1\cdot2}$ and $T_{2\cdot0}$
In a three-buck litter: $S_{1\cdot2}$, $S_{2\cdot0}$ and $T_{1\cdot2}$ or $S_{1\cdot2}$, $S_{2\cdot0}$ and $T_{2\cdot0}$ or $S_{1\cdot2}$, $T_{1\cdot2}$ and $T_{2\cdot0}$ or $S_{2\cdot0}$, $T_{1\cdot2}$ and $T_{2\cdot0}$ — equally divided between all such litters
In a five-buck or more litter: any suitable extension of the smaller distributions

Thus each test litter yields at least one $S:T$ comparison and one b value. Litters of two bucks are used for negative and positive controls only. Intralitter initial weight differences are evenly spread between doses as a precautionary measure against the emergence of a correlation between initial weight and growth response.

The two dosage levels, 1·2 and 2·0 i.u., are not, of course, integral to the scheme; they are purely

arbitrary. The choice of a dosage interval is influenced by the fact that the smaller it is the less frequently will responses fall outside the critical range (this is especially important in assays of materials that are unexpectedly high or low in potency), whereas the larger the interval is the more sensitive and, other things being equal, the more accurate the assays will be. Our values are a working compromise arrived at by accumulated experience coupled with certain dosimetric considerations.

The object of the scheme is to have available at the end of the test the following four, equally weighted, isogenic pair-difference means of growth response:

$(S_{1\cdot2} - T_{1\cdot2})$ and its S.E.$\}$ hence overall $(S-T) \pm \epsilon_{S-T}$
$(S_{2\cdot0} - T_{2\cdot0})$ and its S.E.$\}$

$(S_{2\cdot0} - S_{1\cdot2})$ and its S.E.$\}$ hence overall $b \pm \epsilon_b$
$(T_{2\cdot0} - T_{1\cdot2})$ and its S.E.$\}$

Concentration upon pair differences of response as the metameters *ipso facto* eliminates interlitter variations, and the given arrangement enables this to be done thoroughly despite the limitations and variations of litter size. From the data the assay and the approximate error (incorporating the error of the slope b) can readily be calculated. Furthermore, in a series of assays, fluctuations of b can be followed and any significant differences between the b's of Standard and test material detected.

More than 30 assays have been carried out to date on this plan. In several of the larger ones enough tetrad (four buck) litters were incorporated to permit analysis of variance of growth response and of covariance of initial weight and growth response. Nine such calculations have been made (Table 2).

Note, first, the renewed emphasis on interlitter variations; secondly, that there is further evidence of the negligible influence of initial weight on subsequent growth response; thirdly, that in only one case, that of assay 454, does departure from parallelism approach statistical significance, i.e. the ($P = 0{\cdot}95$) level of variance ratio for the particular circumstances is 4·2, whereas in this assay it is, at most, 4·8; fourthly, that in all cases but one, variations due to slope are highly significant, assay 465 being a borderline exception.

For the purposes of statistical analysis these part assays have each been evaluated from 'internal' estimates of b. In practice, however, this is unsatisfactory. The sampling error of a single-assay estimate of the slope is large. And though the error of an estimate from a number of assays is, of course, correspondingly small, the use of such an estimate would be justified only if it were proven that absolute changes in b did not take place within the covered period of time. In the case of these rats it was found that absolute changes did take place

Table 2. *Analysis of variance and covariance of growth response (GR) and initial weight in nine vitamin-A part-assays composed of isogenic tetrads of buck rats on four different doses, two of standard* (S) *and two of test material* (T)

Assay no.	No. of isogenic tetrads	Calculations based on GR taken (*a*) alone and (*b*) adjusted for init. wt.	Residual σ of GR	Variances of GR due to three specified factors expressed as multiples of the corresponding variances due to residual error: Diff. between litters	Slope b	Departure from parallelism	Coefficient of regression of GR on init. wt.	'Unadjusted' assay as % of 'adjusted' assay	Upper limit of error ($P = 0{\cdot}95$) expressed $\equiv 10$ pairs %
454	11	(*a*)	4·66	2·44	26·76	4·28	−0·563	96·3	140·1
		(*b*)	4·36	2·80	24·73	4·82			143·6
455	17	(*a*)	4·79	3·44	20·77	0·04	−0·125	101·7	150·0
		(*b*)	4·78	3·53	20·95	0·06			149·6
459	7	(*a*)	3·93	13·77	9·19	2·23	−0·123	99·8	160·6
		(*b*)	3·95	13·89	9·74	2·13			158·5
460	9	(*a*)	5·73	2·29	11·78	0·12	−0·201	105·4	151·2
		(*b*)	5·30	1·43	15·84	0·08			141·6
465	9	(*a*)	7·35	2·06	4·35	0·02	−0·115	100·9	206·7
		(*b*)	7·50	2·26	4·04	0·02			211·4
469	11	(*a*)	6·62	3·53	53·17	0·03	+0·297	102·3	133·5
		(*b*)	6·55	3·99	57·54	0			131·1
472	9	(*a*)	7·53	2·25	23·91	0·11	−0·253	105·8	140·9
		(*b*)	7·58	1·55	28·29	0·11			134·9
474	10	(*a*)	6·96	1·78	40·46	1·40	−0·126	100·2	125·8
		(*b*)	7·02	1·63	40·62	1·00			125·7
481	9	(*a*)	4·73	2·11	25·39	1·89	−0·900	97·7	133·0
		(*b*)	4·77	2·13	23·22	1·78			135·5
Weighted means (92 total)		(*a*)	5·89	3·44	24·89	1·05	−0·142	101·1	150·0
		(*b*)	5·85	3·44	25·85	1·05			149·4

and it was therefore thought advisable to use the weighted mean of the estimate of b for any given assay plus those of the few (latterly two) immediately preceding assays: a balance is thereby struck between avoidance of short-term apparent variations of b on the one hand and recognition of real variations over longer periods on the other. Since the introduction of the new regime, the moving averages employed have revealed definite variation trends. All these averages are charted in Fig. 2.

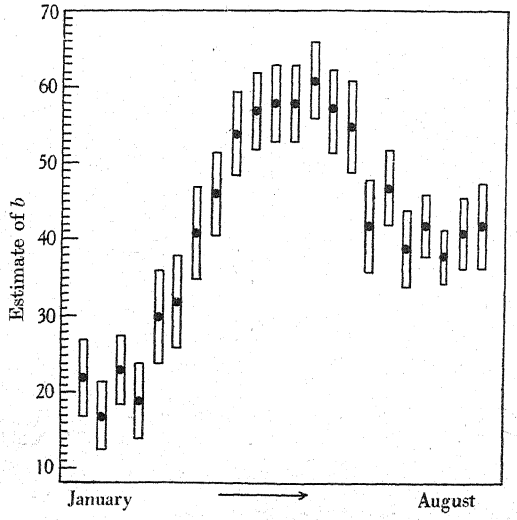

Fig. 2. Variation in b, the constant of slope in the rectilinear growth/log-dosage equation during the current year. Mean and limits of S.E. are given. The whole constitutes an assay-to-assay moving average, each estimate being based on the data of the particular assay together with those of (generally) the two preceding assays—an average of 62 values. (By one 'value' of b is meant the estimate from one isogenic pair difference of response.) The mean interval between assays—and therefore between estimates of b—is 8 days.

It is clear from Fig. 2 that real variations of slope have occurred. If the component series of separate assay estimates is roughly trisected the following averages are obtained (Table 3):

Table 3. *Variations in* b, *the constant of slope*

	Mean b	Standard error
Earliest group (158 values)	24·5	3·23
Middle group (173 values)	57·5	3·43
Latest group (157 values)	42·0	2·95
488 values	41·8	

It is easily demonstrable that any one of these three means is significantly different from the other two. Thus there is danger in using a fixed b over even a few months.

Long-period fluctuations in b are of interest. The mean annual estimates over the past few years (recalculated from the records, because until recently the 'square-growth' b was in use) are given below, together with the responses to 2 units/day and the mean ($P=0·95$) error of a 10-pair assay in which the growth responses to nominally equivalent dosage levels of Standard and test material are equal and the calculated dose-ratio is therefore unity. Under these conditions the error is at a minimum.

Table 4. *Annual fluctuations in certain mean values in vitamin A assays*

Year	Mean growth on 2 units Standard g.	Mean b from isogenic pair-differences only (No. of values)	S.D. of pair-differences of growth $\sigma_{(S-T)}$	Upper limit of error ($P=0·95$) for a 10-pair assay with equal S and T growth responses %
1936	45	48 (141)	7·5	125
1937	51	43 (135)	7·2	127
1938	48	52 (233)	9·6	130
1939	41	36 (107)	10·5	152
1940	38	30 (142)	6·8	138
1941	50	54 (104)	7·0	120
1942 ($7\frac{1}{2}$ months)	41	42 (488)	9·0	136

In the first $7\frac{1}{2}$ months of 1942 the estimation of b has been made from more than twice as many values as in any previous year. Future variations of all important factors will be followed with correspondingly greater accuracy. The highest average error for any one year for ($P=0·95$), 3-weeks, 10-pairs, is 66 to 152 %, which is a little lower than the British Pharmacopoeia error for a 40-pair assay (63–159 %).

Finally, the records of assays carried out on the new plan have yielded information on the comparability of estimates of b from Standard and from test materials. Altogether there were 24 assays, involving 416 values, in which β-carotene Standard was compared with test-materials in which vitamin A itself supplied the whole of the vitamin A activity (Table 5).

Table 5. *Comparison of estimates of slope from response to β-carotene and to vitamin A*

	No. of values	Mean b	S.E.	Variance	Degrees of freedom n
β-Carotene (S)	221	43·6	2·69	1601	197
Vitamin A (T)	195	39·6	3·02	1781	171
Difference		4·0	4·05		

Variance ratio $=1·112$. $z=\frac{1}{2}\log_e 1·112=0·0531$.
S.E. of $z=\sqrt{\{\frac{1}{2}(1/n_S+1/n_T)\}}=0·0739$.

Neither the two b's nor the variances are significantly different; thus an important condition of satisfactory assay work is fulfilled. This conclusion depends to some extent on the mean Standard: test-

material dose-ratio of the component assays, because in a series in which the mean dose-ratio is much higher or lower than unity a systematic difference due to departure from parallelism may invalidate the result. The mean dose-ratio of the above 24 assays is sufficiently close to unity, being approximately 1·1.

SUMMARY

(i) For the vitamin A-test rats in this laboratory interlitter variations in response are large enough to justify strict adherence to isogenic comparisons. The ratio of the numbers of isogenic or anisogenic pairs required for equal accuracy is about 1 : 2.

(ii) A new assay plan for routine work is described. A moving average estimate of b, the constant of slope, is employed.

(iii) Real variations in b are found to occur over comparatively short periods.

(iv) The b's for vitamin A and β-carotene are shown to be substantially similar.

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Further Studies of Cryptogam Tissue Proteins: Protein Composition and Tissue Function in Plants

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In an earlier article [Lugg, 1940] the amide and some amino-acid contents of the photosynthesizing tissues of *Lunularia cruciata*, *Pteridium aquilinum* and *Selaginella uncinata*,* and similar data for leaf protein of *Trifolium subterraneum*, were reported. It was pointed out that the amide, tyrosine and tryptophan contents revealed no marked changes in the compositions of the proteins to correspond with the different degrees of evolutionary development of the three cryptogams and of the phanerogams (i.e. *Trifolium subterraneum*) which have been examined.

In this article, the results of further analytical work with the same protein preparations will be reported. Some data are given for one of the extracted protein preparations from pinnae of *Pteridium aquilinum*, and the protein in the thalli of *Ulva lactuca*.

METHODS

The protein preparations. With the exception of the *Ulva lactuca* preparation, the protein preparations and the tissues from which they were obtained, have been described [Lugg, 1940].

The *U. lactuca* preparation was made from thalli collected on 11 April 1941 from a rocky ledge in the Southern Ocean near Port Elliot. The ledge was completely submerged at high water but merely washed over by the waves at low water. The thalli, thoroughly wet with sea water, were examined about 36 hr. after collection. They did not differ in general appearance from freshly collected material, nor appear more flaccid. They were rinsed with distilled water and pressed between sheets of blotting paper. After being weighed, they were dried at 65° in a current of air and then powdered, prior to removal of non-coagulable and other non-protein nitrogenous substances [Lugg, 1939]. Complete drying was effected by heating at 105° for about 8 hr. and exposing at low pressure over H_2SO_4. Nitrogen estimations were made by the Kjeldahl method.

The fresh thalli contained 15·8 % dry material consisting of 1·18, 0·90 and 0·83 % of 'total', 'coagulable' and 'protein' N respectively. The dried 'whole protein preparation' contained 2·04 % N, and 11·5 % ash (by ignition in air at about 600°). The ash contained large amounts of $CaSO_4$.

Partial analyses of the protein preparations. The

* Previously reported as '*Selaginella* sp. (probably Jungermannioides group)'. I am indebted to Prof. J. G. Wood for completing the identification of this plant. *S. uncinata* is not in the Jungermannioides group.

amide, tyrosine, diiodotyrosine, tryptophan, cystine plus cysteine and methionine contents of the protein preparations were estimated by methods described in earlier articles [Lugg, 1937; 1938 *a*, *b*, *c*]. The arginine, histidine and lysine contents of one of the extracted protein preparations from pinnae of *Pteridium aquilinum*, were estimated by Tristram's [1939] methods. From tests which have been recorded with the descriptions of these methods of estimation, it seems probable that the considerable impurities in the protein preparations would not have vitiated the results obtained (Table 1). Estimations by the other methods (Table 2) are vitiated to some extent by impurities such as carbohydrates, and sometimes by small amounts of prosthetic carbohydrate. In both tables the amino-acid contents have been corrected for such losses as the pure free amino-acids undergo in the procedures (including hydrolysis) employed in the estimations [Lugg, 1938 *a*, *b*; Tristram, 1939].

In Table 1 are shown, in the order of the columns, the characteristic label of the preparation consisting of a number followed by W, E or R, signifying respectively that the preparation was made from whole tissue, a tissue extract, or from tissue residues after extraction of some of the protein, followed by the name of the plant and tissue from which the preparation was derived, and the analytical data.

Most of the amide, tyrosine, diiodotyrosine and tryptophan contents have been already reported [Lugg, 1940], and the preparations are numbered as before. The *Ulva lactuca* preparation has the number 0. The numbers are in the order of what is customarily accepted as the increasing degree of evolutionary development of the plants.

Table 1. *Partial analysis of the plant-protein preparations by the method of Lugg* (*see p. 133*)

	Preparation (see text)	Total N (%)	% total N: Amide	Tyr.	Diiodo-tyr.	Try.	Cyst.	Meth.	Cyst. + meth.	SO_4-S ×100/N	Ash
0W	*Ulva lactuca* (thalli)	2·04	5·59	1·34	0·0	1·01	2·2	1·2	3·4	124·5	11·5
1W	*Lunularia cruciata* (thalli)	3·92	5·52	2·43	0·0	1·70	1·31	1·36	2·67	0·51	3·5
2W	*Pteridium aquilinum* (pinnae)	5·82	5·12	2·43	0·0	1·70	1·31	1·36	2·67	0·51	3·5
2E	,,	11·1	4·97	2·08	—	1·13	1·11	1·71	2·82	0·02	1·4
2R	,,	2·70	5·14	2·20	—	1·13	1·20	1·65	2·85	0·37	1·6
2E1	,,	12·0	4·92	2·14	0·0	1·19	1·16	1·74	2·90	0·06	1·3
3W	*Selaginella uncinata* (leaves and stems)	3·12	5·31	2·60	0·0	1·43	1·22	1·49	2·71	0·64	9·9
4W	*Trifolium subterraneum* (leaves)	7·17	5·29	2·53	—	1·75	1·15	1·32	2·47	0·08	0·7
4E	,,	14·1	5·35	2·64	—	1·62	1·13	1·38	2·51	0·00	0·4
4R	,,	5·26	5·39	2·64	—	1·67	1·12	1·29	2·41	0·00	3·1

Estimations unaffected by presence of carbohydrate. Amino-acids are given as percentage of total N.

Percentage errors, from replicate estimations in present and earlier work; N in dry preparation 0·5, amide 1·5–2, tyrosine 2–3, tryptophane 3–4, cystine + cysteine 3–5, but 20 for 0W, methionine 4–6, but 30 for 0W; sum of last two 2–3, but 10 for 0W. The error for arginine, histidine and lysine estimations from the work of Tristram [1939].

Table 2. *Partial analysis of the protein preparations by other methods* (*see p. 133*)

	HI digestion and titration: Cyst.	Meth.	Tristram [1939] (see p. 134): Arg.	Hist.	Lys.
2E1	1·03	1·80	13·5*	2·4	6·45
4E	0·93	1·16	—	—	—

Estimations affected by the presence of carbohydrate and not corrected for the effects.

Amino-acids are given as percentage of total N. Corrections were applied according to Tristram [1939]. Arginine +2·6 mg. N in addition to solubility correction of Ag. arginine. Histidine + 0·3 mg. N. Lysine + 1·6 mg. N. Hydrolysis factors 1·06, 1·03 and 1·07 respectively.

* This value increased to 15·3 when corrected for effects of presence of carbohydrate impurity.

DISCUSSION

From comparison of the partial compositions of 'whole', 'extracted' and 'residual' protein preparations (Table 1), the conclusion was drawn that 2E, 2E1 and 4E were reasonably representative of the 'whole' proteins in the tissues from which they were extracted, and the analyses shown in Table 2 are, therefore, assumed to be analyses of representative proteins, but they are undoubtedly subject to systematic errors, because such preparations contain impurities of a carbohydrate nature (e.g. pentosan or mucilage).

Lugg [1938*b*] found that these impurities affected the estimations of cyst(e)ine and methionine by the HI digestion procedure differently with different protein preparations and that the differences were

irregularly related to the amounts of impurity*, the standards of reference being estimations by the differential oxidation procedure. Thus corrections could not safely be attempted, and the results are only imperfect checks upon the values obtained by the less specific differential oxidation procedure. Note that the methionine content of 2E 1 was actually slightly higher when estimated by the former procedure than by the latter. This may have been due to the presence of an impurity in the HI digest capable of reacting as methionine does in the titration procedure, but random errors could easily account for the discrepancy. Nearly all the cyst(e)ine plus methionine estimated by the differential oxidation procedure could be accounted for by the HI digestion procedure in this instance, which is unusual with preparations as impure as 2E 1 appears to be (see below).

Tristram [1939] found that the influence of these impurities upon the estimations of histidine and lysine was irregularly related to the amount of impurity, but was small when the latter was not overwhelmingly large. For amounts of impurity such as those in 2E 1, he considered the effects too small and uncertain to justify corrections. He found, however, in the estimation of arginine that the effects of small amounts of impurities were sufficiently regular to warrant the use of a correction factor calculable from the estimated percentage impurity.

Two methods of estimating the amount of impurity in preparations like 2E 1, or of estimating the percentage N in the hypothetically 'pure' proteins, have been discussed elsewhere [Chibnall, 1939]. They are (1) computation from extensive amino-acid analyses of an impure preparation, and (2) calculation from the amount of humin precipitated at *p*H 2·5 after acid hydrolysis on the assumption that the impurity has behaved like a pentosan. These methods lead to substantially the same results when applied to extracted preparations from *Dactylis glomerata*. Since the leaf proteins of all phanerogams so far examined have very similar compositions, there is some justification for the assumption that the pure proteins all contain about 17 % N. The pinna protein of *Pteridium aquilinum* has not been sufficiently examined to permit the use of method (1) except in a very loose way, i.e. since from Table 1 it corresponds fairly closely in composition with the leaf proteins of the phanerogams examined, it too probably contains about 17 % N, if pure. Data for method (2) were obtained from both 2E and 2E 1, which yielded respectively, 28·2 and 24·8 % of 'humin', containing 7·4 and 6·9 % of the protein N. Calculations from these data (ignoring ash) suggest that the 'pure' preparations would have contained 17·8 and 17·4 % N respectively and it seems reasonable to accept the rounded value, 17 %. The actual preparation must have contained 30 % impurity, and from Tristram's [1939] correction table, the correction factor to be applied to the arginine content reported in Table 2 is 1·20/1·06. The corrected value is shown in a footnote to the table.

In Table 3 the partial composition of the proteins in the somatic photosynthesizing tissues of the cryptogams and those of various phanerogams are compared. The arginine estimations are corrected for impurities. The plants are grouped into families, orders, classes and divisions in the natural classification. The divisions have been arranged from left to right in the sequence of increasing evolutionary development, and the sequence has been maintained, as far as current evolutionary theory permits, down to the genera. Some of the extracted protein preparations are known not to have been fully representative of the tissue proteins, but they probably did not differ from them very much. These data have been drawn from material in the present article and from analyses by Lugg [1938*c*; 1939] and Tristram [1939]. Mean values are quoted where analyses have been made of more than one preparation from any particular plant species.

The striking feature of Table 3 is that among representatives of the bryophyte, pteridophyte and spermatophyte divisions, differences in tissue protein composition are small, the greatest variability being in the tryptophan content. The tissue protein of *Pteridium aquilinum* has a lower tyrosine and tryptophan content than those of the other plants belonging to these three divisions, and as compared with these its methionine content is rather high relative to its cyst(e)ine content; but in its amide, arginine, histidine and lysine content it is very similar to the others.

Unfortunately the cystine and methionine contents of the tissue protein of the only representative of the thallophyte division (*Ulva lactuca*) are subject to considerable errors, owing to the 2·5 % of sulphate-S in the preparation. This was present possibly as sulphuric acid esters of substances of carbohydrate nature. The possibility of the presence of nitrogenous substances other than protein, in preparations like 0 W, cannot be ignored. However, the rate of production of NH_3 by hydrolysis (in the amide-N estimation) was very similar to those found for phanerogam leaf-proteins [Lugg, 1938*c*], and the presumption is that non-protein nitrogenous substances had been removed completely. The cystine content of the tissue protein is rather high and the tryptophan rather low, in comparison with those of the other proteins, and though there is nothing unusual in the amide content, the tyrosine

* Smith & Wang [1941], ignoring these complications, have contended from their analyses of pasture protein preparations by this procedure, that the protein composition varies somewhat with season.

content is decidedly low. Many marine algae (notably members of the class Phaeophyceae) contain fairly large amounts of iodine compounds, but this *U. lactuca* preparation (like the other preparations examined) contains no diiodotyrosine.

bility than are revealed in Table 3. It is not known whether the composition of whole proteins of cryptogam tissues are as independent of climate, locality of growth, manurial treatment and tissue age, as are those of the phanerogams. Small varia-

Table 3. *Partial compositions of the proteins of the somatic, photosynthesizing tissues of plants*

Division	Thallophyte	Bryophyte	Pteridophyte		Spermatophyte				
Subdivision	Algae	Hepaticae			Angiospermae				
Class	Chlorophyceae				Monotocyledon		Dicotyledon		
Order	Ulvales	Marchantiales	Filicales	Lycopodiales	Glumiflorae		Centrospermae	Rosales	
Family	Ulvaceae				Gramineae		Chenopodiaceae	Leguminosae	
Genus and species	*Ulva lactuca*	*Lunularia cruciata*	*Pteridium aquilinum*	*Selaginella uncinata*	*Dactylis glomerata*	*Lolium perenne*	*Beta cicla*	*Trifolium subterraneum*	*Trifolium pratense*
Amide	5·59	5·52	5·12	5·31	5·07	5·22	5·28	5·39	*5·40*
Tyrosine	1·34	2·43	2·06	2·60	2·48	2·45	2·63	2·53	*2·50*
Diiodotyrosine	0·0	0·0	*0·0*	0·0	*0·0*	—	—	—	—
Tryptophan	1·01	1·70	1·14	1·43	1·84	1·84	1·50	1·75	*1·70*
Cyst(e)ine	2·2	1·31	1·14	1·22	1·40	1·44	1·45	1·15	*1·25*
Methionine	1·2	1·36	1·63	1·49	1·54	1·53	1·43	1·32	*1·29*
Arginine	—	—	*15·3*	—	*14·6*	*14·0*	*12·8*	—	*14·9*
Histidine	—	—	*2·4*	—	*2·4*	*2·4*	*2·25*	—	*2·3*
Lysine	—	—	*6·45*	—	*6·1*	*6·0*	*6·7*	—	*6·5*

Estimations corrected where necessary for carbohydrate impurities. Values in italics are for extracted protein preparations: those in ordinary type for whole protein preparations.

Amino-acids are given as percentage of total N.

Little work has been done with cryptogams other than unicellular organisms like yeasts and bacteria. Mazur & Clarke [1938] studied the amino-acid composition of the nitrogenous substances not extracted by alcohol from the tissues of various marine algae. In thalli from one specimen of *U. lactuca* they found that 5·5 % of the remaining N was amide-N; and in this and another specimen, that 0·0 and 0·0 % was tyrosine-N, 0·3 and 0·6 % was tryptophan-N, 1·8 and 2·1 % was cyst(e)ine-N, and 0·0 and 0·0 % was methionine-N. Except for the amide and cyst(e)ine content, these results are very different from those reported in Table 1. Of the N remaining after alcohol extraction in tissues of *Osmunda claytoniana* (pteridophyte), the same authors found 0·6 % cyst(e)ine-N, 1·1 % methionine-N, 7·6 % arginine-N, 3·7 % histidine-N and 3·6 % lysine-N. These results again differ considerably from the values in Table 1. Mazur & Clarke's analyses were actually analyses of the nitrogenous substances dissolved out by hot formic acid solution from the alcohol-extracted tissues, and it is not certain that the formic acid dissolved out unaltered protein.

The amounts of those amino-acids which were not estimated may exhibit different degrees of variations have been reported by Chibnall [1939], Lugg [1938 *a*, *b*; 1939] and Tristram [1939] for the whole proteins of the leaves and for preparations believed to be fairly representative, but in regard to age of tissue the sources of material included neither seedlings at the one extreme nor senescent plants at the other. Barrien & Wood [1939] and Hanson, Barrien & Wood [1941] have shown that the N : S ratios in their protein preparations from Sudan grass (*Andropogon sudanensis*) were higher for seedling leaves than for older leaves. Their S estimations, however, included any sulphate-S which may have been present. Reference has been made above to a claim by Smith & Wang [1941], of variation with season, but there is, as yet, no conclusive evidence that the composition of the whole leaf protein of any phanerogam varies markedly with changes in the conditions enumerated.

Phanerogam tissues contain at least two proteins of somewhat different composition. These have been tentatively referred to the fluid protoplasm and vacuole contents on the one hand, and to the granular inclusions like chloroplasts, chondriosomes and nuclei on the other [Lugg, 1938 *a*, *b*; 1939]; or to chloroplasts on the one hand, and the rest of the protein-containing morphological units on the

other [Barrien & Wood, 1939; Hanson *et al.* 1941]. Chibnall [1939] showed that vacuole protein may differ somewhat in composition from the proteins of the fluid and granular parts of the protoplasm.

In these somatic, photosynthesizing tissues two well-defined types of process occur—the ordinary living processes of the cell, and those processes intimately connected with photosynthesis—and it is possible that the physical and chemical properties and compositions of the proteins associated with these processes vary very little. Perhaps also the proportions of the different proteins differ little in different species, different climates, with different ages of tissue and so on.

It is generally agreed that present-day bryophytes, pteridophytes and spermatophytes represent increasing degrees of evolutionary development from a common ancestor; and there is no reason for supposing either that the basic living processes, or that the photosynthesizing processes, have changed radically, either in the gametophyte or the sporophyte generations of the plants. These considerations are in harmony with the observation recorded in this article, namely, that the 'whole' proteins of the gametophyte tissue of bryophytes, and of the sporophyte tissue of pteridophytes and spermatophytes, appear to be of similar composition. It is generally accepted that the Chlorophyceae (Thallophyte) are in the same main branch of evolutionary descent as the green land-plants [Bower, 1929; 1935; Fritsch, 1929]. It is likely that the *Ulva lactuca* thalli belong to both the sporophyte and the gametophyte generations of the plant [Föyn, 1929]. Possibly the individual proteins in the thalli of *U. lactuca* are similar to those of these other plants, but occur in very different proportions. The radical evolutionary step by which the tissues were transferred from a water to an air environment may have demanded modification of the composition and physical properties of individual proteins. It would be of interest to discover, from analyses of tissue proteins of sea phanerogams, whether the reverse step has required any modification of protein composition.

In sharp contrast to what appears to be the relative uniformity of composition of 'whole' proteins of the somatic photosynthesizing tissues of the bryophytes, pteridophytes and spermatophytes, is the non-uniformity of composition of proteins in tissues characteristic of the spermatophytes alone, namely, the seeds. Since the seedling depends for its early growth upon the seed proteins, these should correspond fairly closely in composition with the leaf proteins. This is sometimes not the case. True, satisfactory analyses have been made only of the proteins of angiosperm seeds, and these appear to be elaborated in cells possessing $3n$ chromosome nuclei, but in regard to tissue protein composition no particular significance can be attached at present to chromosome number (cf. tissue protein composition of *Lunularia cruciata* possessing n chromosome nuclei, with those of the pteridophytes and spermatophytes possessing $2n$ chromosome nuclei). Leaf proteins appear to be of 'fibrous' type and are very readily denatured, whereas many seed proteins are of 'globular' type and can withstand prolonged periods of partial desiccation at relatively high temperatures. Possibly biological fitness for survival might otherwise be assured by fairly close correspondence of composition, whereas denatured seed proteins may be of little use to young seedlings; resistance to denaturation of seed proteins may be almost a prime criterion of fitness at the present stage of evolutionary development of the spermatophytes, an unfavourable composition of the proteins from the point of view of seedling nutrients being in comparison a small disadvantage.

The comprehensive analysis of a single protein preparation is laborious, and there are many species of pasture plants. The object of the investigation here described was to extend, if possible, the basis of the belief that for the purposes of animal nutrition studies, comprehensive analyses of pasture proteins might well be confined in the first instance to those of only a few species representative of the most important families of pasture plants. It is considered that the investigation has substantially broadened the basis for that belief.

SUMMARY

Partial analyses have been made of the 'whole' proteins of the somatic, photosynthesizing tissues of a variety of cryptogams (a thallophyte, *Ulva lactuca*; a bryophyte, *Lunularia cruciata*; and two pteridophytes, *Pteridium aquilinum* and *Selaginella uncinata*), and also of one more phanerogam (*Trifolium subterraneum*).

The apparent similarity of composition of tissue proteins of plants from the bryophytes upward through divisions exhibiting increasing degrees of evolutionary development, has been contrasted with the pronounced lack of uniformity in the compositions of seed proteins. An attempt has been made to interpret the findings in terms of hypothetical relationships between tissue function and environment, and protein composition and physical and chemical properties.

The assistance of Mr R. A. Weller is gratefully acknowledged. Thanks are due also to Miss B. S. Barrien and Miss C. M. Eardley for the collection and classification of plant material and for helpful discussions.

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A Simple Fluorescence Comparator and its Application to the Determination of Porphyrin

By C. RIMINGTON, *With an addendum by* E. Schuster, *From the National Institute for Medical Research, London, N.W. 3*

(*Received 13 October 1942*)

Several substances of biochemical interest are either themselves fluorescent in radiation of suitable wavelength, or are readily transformed quantitatively into fluorescent materials. Among these may be mentioned the porphyrins, stercobilin and related pigments, flavins, including vitamin B_2, certain chemotherapeutic drugs and aneurin.

Quantitative methods based on the measurement of fluorescence intensity have the advantages of being highly specific and extremely sensitive, enabling very small quantities of material to be determined with accuracy once they are suitably extracted. Such methods suffer, however, from the disadvantage of requiring, for accurate results, relatively expensive apparatus such as the combination of an ultraviolet source with the Zeiss or Pulfrich photometer or some type of photoelectric colorimeter.

For the purposes of a particular investigation it became necessary to carry out a number of quantitative determinations of porphyrin in single specimens of urine, each containing about 2 μg. of porphyrin. Only measurements of fluoresence intensity were sufficiently sensitive, and in order to bring the method within the reach of the small medical or factory clinic an inexpensive fluorimeter or fluorescence comparator was essential which, acting very much like an ordinary colorimeter, would give results of sufficient accuracy for general purposes. It was realized that, with suitable adjustments, the instrument would serve for the determination of a number of fluorescent materials in addition to porphyrins, e.g. vitamins, drugs etc., as noted above. Its construction, and operation with porphyrin solutions, will be described in the present paper, whilst other applications will be dealt with in subsequent communications.

Requirements of a fluorescence comparator

The essential requirements of a fluorescence comparator are:

(1) An easily operated source of ultraviolet radiation with suitable filter to remove visible light.

(2) An optical system to conduct the visible fluorescent light into the eyepiece. This should not be in the same axis as the ultraviolet source. The emergent fluorescence is collected in the present instrument at an angle of 55° to the ultraviolet beam and is reflected by a system of mirrors into the eyepiece, the optical path being approximately equal to the distance of normal vision, so that lenses may be dispensed with. A suitable filter is interposed, the transmission of which covers a suitably narrow band in the region in which the fluorescence lies. With porphyrins, Ilford spectral filter no. 607 (orange) transmitting 595–650 mμ is used.

(3) A device for altering the intensity of one beam. In the present instance glass screens made by photographing a standard neutral-tinted wedge are used, and by adopting a reciprocating arrangement, rotation of a graduated knob brings about a gradual, but uniform, darkening of the field emanating from the standard. Matching is performed as in a colorimeter.

The construction and mechanical arrangements of the apparatus are described in the Addendum by Dr E. Schuster who has been responsible for the detailed design and who has kindly constructed the first four models produced.

METHODS

Calibration and operation of the apparatus for the determination of porphyrin

The extraordinarily intense fluorescence of porphyrin solutions has been studied by a number of workers from Dhéré [1933] onwards, one of the most detailed of recent investigations being that of Fink & Hoerburger [1933] in which the effects of *p*H, salt concentration, temperature etc. were described. These authors showed that porphyrin isomers could be recognized by differences in their *p*H-fluorescence curves.

Fikentscher [1932] first described a method of porphyrin determination based upon the measurement of fluorescence intensity in a Zeiss step-photometer, and in his article will be found a consideration of the factors governing the accuracy of any such method. With concentrations of from 0 to 10 μg. porphyrin/ml. fluorescence intensity is proportional to concentration (other things such as incident illumination remaining constant), but above this limit quenching intervenes.

An attempt to reduce the cost of the necessary apparatus has been made by Stevens & Turner [1937] who describe a fluorescence method in which a carbon arc serves as the source of ultraviolet radiation, and the fluorescence elicited is measured by an electrical circuit containing two photo-cells.

A much more convenient and relatively inexpensive source of ultraviolet radiation is the 'Osira' mercury discharge lamp with Wood's glass bulb, which is incorporated in the present machine. As fluorescent standard, a solution of coproporphyrin in 5% HCl is employed in a sealed Monax glass test tube, the concentration being 2 μg./ml. This is made up from the tetramethyl ester as follows: A stock solution is first prepared by dissolving 1·085 mg. of solid coproporphyrin tetramethyl ester (equivalent to 1 mg. free coproporphyrin) in 5 ml. of glacial acetic acid, and adding 5% HCl (137 ml. conc. HCl to 1 l.) to a total volume of 100 ml. This stock solution contains 10 μg./ml. and should be kept in the dark in a stoppered bottle. The acetic acid it contains increases its stability. Solutions of lower concentration are prepared from it by dilution, as required, and in such a way that the final concentration of HCl is 0·25% (see later). Fikentscher reports that his standards (0·375–0·75 μg./ml. made up in 5% HCl without acetic acid) suffered no change in 6 weeks.

Attempts to find a stable fluorescent dye to serve as an artificial standard in place of porphyrin have not been successful. An ideal standard, in addition to possessing stability, should both absorb at the same wave-length in the ultraviolet, and fluoresce in the same spectral region, as does coproporphyrin. Several substances have been investigated, one of the most promising being resorufin (alkali salt), but the intensity of its fluorescence is such that in concentrations sufficient to match 2 μg. porphyrin/ml. quenching occurs. Safranine, the use of which was kindly suggested to me by Mr Jope of the Nuffield Laboratory, Oxford, also exhibits quenching at concentrations higher than about 30 mg./l., corresponding in fluorescence intensity to 0·3 μg. coproporphyrin/ml.

A coproporphyrin standard which has been in regular use in one apparatus in a sealed tube has shown no deterioration during nearly 3 months.

Monax glass test-tubes are used for both standard and unknown solutions. This glass does not fluoresce in ultraviolet radiation and is moderately transparent. Other suitable makes such as Hysil (for which suggestion I am indebted to Mr Jope) may also be employed.

To calibrate the instrument the standard 2 μg./ml. porphyrin solution in its sealed tube is placed in the right-hand side of the instrument and varying dilutions of porphyrin in similar test tubes in the left-hand side; sets of 5 or 10 matchings are then recorded for each dilution. With the instruments we have constructed a plot of the scale reading against the porphyrin concentration exhibits a linear relationship (see Fig. 1). Since accuracy of matching is greater at the lower intensities, it is best to dilute a more concentrated solution with 0·25% HCl until it contains about 0·25 μg./ml. which affords a convenient intensity of fluorescence. The range of the instrument may be taken as being from 0·05 to 0·5 μg. porphyrin/ml.

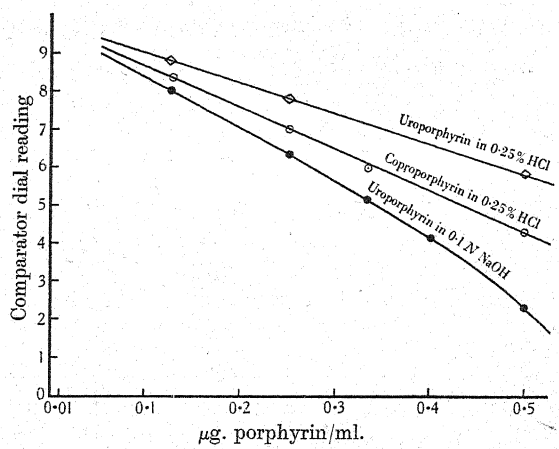

Fig. 1. Calibration curves of fluorescence comparator for porphyrin.

A porphyrin solution of unknown concentration is examined in exactly the same way, the mean of 5 or 10 matchings being accepted and the corresponding porphyrin concentration read off from the calibration graph.

As in all colorimetric work, it is advantageous to work in the dark. The sensitivity of the human eye to differences in colour intensity (especially reds) is known to vary considerably, but there is no doubt that any particular observer improves with practice.

Relation between pH and intensity of fluorescence

Fink & Hoerburger [1933] investigated the fluorescence of a variety of porphyrins both natural and synthetic, using buffer solutions to cover a *p*H range of from about 2·0–8·0, and found that, in general, fluorescence is minimal in the region of the isoelectric point, but increases progressively as the solution becomes more acid or more alkaline. An exception was found in the case of the aetioporphyrins which possess no carboxyl groups and fail to show the increase of fluorescence in alkaline solutions. The conclusion was drawn, therefore, that it is the + or − charged porphyrin molecule which is responsible for fluorescence.

As stated above Fink & Hoerburger's [1933] investigations did not extend to solutions more acid than *p*H 2 nor more alkaline than *p*H 8. Fikentscher [1932] had previously, and somewhat arbitrarily, selected 5 % HCl as the medium to be used as solvent.

Using the fluorescence comparator described in this paper and a fixed standard, it was at once apparent that fluorescence in 5 % HCl is much less than maximal and that small variations in acid concentration in this region result in relatively large discrepancies.

The *p*H fluorescence curves of coproporphyrin (I) and uroporphyrin (III) were explored from the isoelectric point to regions of high acid concentration as follows: Stock solutions of the porphyrins were made up from the esters in 5 % HCl containing 5 % acetic acid as previously described, the uroporphyrin concentration being 20 μg./ml. and the coproporphyrin 10 μg./ml. It may be noted here that 5 % HCl is the most convenient strength of acid to use in preparing solutions from the esters; the pigment dissolves in this solvent in presence of acetic acid and hydrolysis of the ester takes place slowly on standing at room temperature. Na citrate-HCl buffers [Clark, 1928] to cover the range *p*H 4·95–1·17, whilst for still more acid solutions, unbuffered HCl of concentrations 0·5, 2·5, 5 and 10 % were used. 0·1 % acid afforded a useful comparison with the buffered solutions. 0·1 ml. quantities of the stock uroporphyrin or 0·2 ml. of coproporphyrin were diluted to 5 ml. (final concentration 0·4 μg./ml.) with the appropriate buffer, the *p*H of the solution being measured by the glass electrode and its fluorescence by the comparator. In the case of uroporphyrin (III) the investigation was extended to include the region on the alkaline side of the isoelectric point by means of Na citrate-NaOH buffers. The results are shown in Figs. 2 and 3.

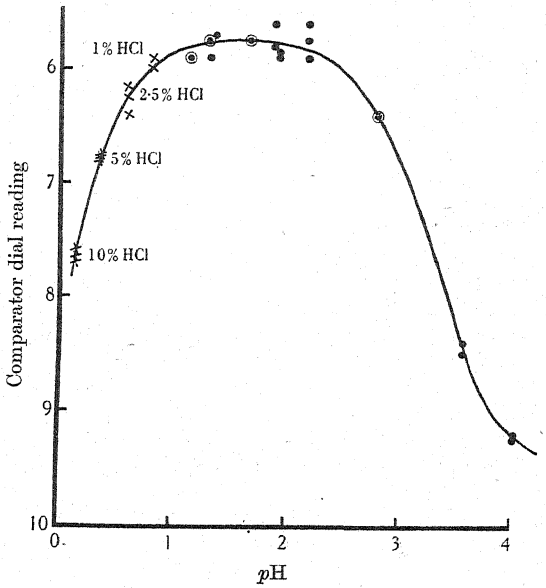

Fig. 2. Fluorescence intensity of coproporphyrin in acid solutions. Porphyrin concentration 0·4 μg./ml. • Buffered solutions. × Unbuffered solutions. ⊙ Coincident values.

The intensity of fluorescence of both porphyrins rises to a maximum at about *p*H 1·4, but declines rapidly as the concentration of acid is increased.

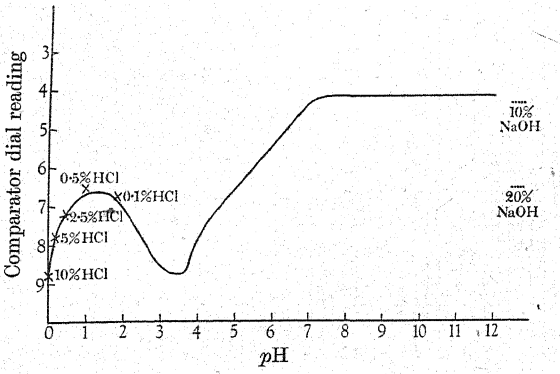

Fig. 3. Fluorescence intensity of uroporphyrin in acid and alkaline solutions. Porphyrin concentration 0·4 μg./ml.

5 % HCl is seen to be a most unsuitable solvent for quantitative measurements, whereas in the region *p*H 1·2–2·0 for coproporphyrin, or 1·0–1·6 for uroporphyrin, fluorescence intensity remains practically constant. The acid number of coproporphyrin is fortunately low, viz. 0·1, so that a solvent such as 0·25 % HCl having *p*H 1·24 can be used to extract it directly from ethereal solution.

On the alkaline side the fluorescence of uroporphyrin increases regularly from the isoelectric point to a *p*H of about 8·0 after which it remains constant until *p*H 12 (0·1 *N* NaOH) is reached. Even in *N* NaOH the intensity is substantially the same, but in 10 % NaOH it has dropped to about one-half, and in 50 % alkali it is practically extinguished.

In high concentrations of both alkali and acid, therefore, the intensity of porphyrin fluorescence is depressed. These changes are reversible, the porphyrins being relatively very stable molecules, and it seems reasonable to suggest on the basis of Fink & Hoerburger's [1933] theory that the fall in fluorescence intensity is due to suppression of the ionization of the molecule in presence of the overwhelming concentration of OH or H ions.

A practical outcome of these investigations is the suggestion that quantitative fluorescence intensity measurements for both uroporphyrin and coproporphyrin should be made in 0·25 % HCl (*p*H approx. 1·24) when small variations in *p*H will have relatively little effect upon the result. In the case of uroporphyrin it is convenient under certain circumstances to work in alkaline solution and for this purpose 0·1 *N* NaOH is a suitable solvent to employ. The fluorescence comparator has been calibrated for all these possibilities.

Equal quantities of uroporphyrin (I) and uroporphyrin (III) under the optimum conditions exhibit equal intensity of fluorescence. It should clearly be possible, however, to employ the instrument for identification of porphyrin isomers by plotting the *p*H-fluorescence curves according to Fink & Hoerburger in a series of buffer solutions on either side of the isoelectric point.

Preparation of the porphyrin extract from urine

In this paper the manipulation of the fluorescence comparator has been described first, since it is desired to emphasize that its function is to record the concentration of any given solution. Errors introduced in the preparation of the extract, variations in porphyrin excretion during the day etc., must not be confused with those inherent in the use of the comparator.

Little is known concerning the level of porphyrin excretion throughout the day, hence wherever possible a 24-hr. specimen should be collected and an aliquot portion of 25–50 ml. taken from this for analysis.* One-tenth of the volume of glacial acetic acid is then added, followed by 2 vol. of solvent prepared by mixing 9 vol. of ether with 1 vol. of light petroleum (B.P. 40–60°) and the mixture is shaken in a stoppered separatory funnel for about 1 min. The addition of petroleum minimizes the risk of emulsification whilst having no appreciable effect upon the extraction of the porphyrin. When separation is complete the lower layer is drawn off and discarded, and the ethereal layer is washed three times in succession with about an equal volume of distilled water. The porphyrin is now transferred to 0·25 % HCl by adding 2 ml. of acid, shaking and transferring carefully to a Monax test tube graduated at 5 and 10 ml., then repeating the extraction with 1·5 ml. and lastly with 1 ml. of acid. The pooled acid extracts are then made up to the 5 ml. mark, the tube stoppered and the contents mixed. It is then placed in the left-hand side of the fluorescence comparator and a set of readings made. Reference to the calibration graph gives the concentration of porphyrin/ml. of extract. Should the solution require further dilution, it may be made up to the 10 ml. mark with 0·25 % HCl. From start to finish a complete determination can be made in about 15 min. The used solvent may be collected for eventual recovery.

It will be noticed that only a single extraction of the urine with ether is performed. This is justifiable for a clinical method on grounds of speed, convenience, economy and the degree of complete extraction obtained. To perform a second extraction would mean the use of a second separatory funnel or an intermediary vessel to store the ether, and the use of twice the amount of solvent. Using solutions of pure coproporphyrin containing from 0·05 to 0·5 μg.

* Mr Jope of the Nuffield Laboratory, Oxford, has informed me in a private communication that he finds the urinary porphyrin excretion may vary widely throughout the course of the 24 hr.

Table 1. *Comparison of porphyrin determinations on pathological urines by the standard method and the fluorescence comparator method*

Case	Diagnosis	Standard method μg. porphyrin/l.	Fluorescence comparator μg. porphyrin/l.	Difference %
Th.	Toxic jaundice	79·3 } 89·7* }	75	− 5·4 −18·5
Shep.	,,	319*	320	+ 0·3
Do.	Sprue	102	100	− 2·0
		25·2	25	− 0·8
Co.	Aplastic anaemia	70·8*	60	−15·3
Sh.	,,	111*	70	−36·9†
Sh.	,,	58*	60	+ 3·5
V.d.S.	Hepatic insufficiency	156	167	+ 7·1
		70	77	+10·0
Sm.	Hepatic cirrhosis	47·8	43	−10·0
Thom.	Addison's disease	77·7	77	− 0·9
Ber.	Acute idiop. porphyria	313	306	− 2·2
Ber.	,,	769	750	− 2·5
Ber.	,,	916	1080	+17·9
Res.	Pellagra	892·5	768	−13·9

* Determinations made by Dr J. Kench, Royal Infirmary, Manchester.

† The acid extract was much coloured by foreign pigment (from a highly pigmented urine) which probably led to a high spectrophotometric result.

of porphyrin/ml., i.e. from approximately the normal to ten times the normal concentration in urine, and subjecting them to the extraction procedure outlined above, it has been found that the recovery is about 90 %. Should turbidity affect the final acid extract, it may be centrifuged.

Agreement with the standard procedure

In order to test the reliability of the fluorescence method when applied to normal and pathological urines, a number of specimens were analysed by this technique and also by the standard procedure of Fischer, in which to 1 l. or more of urine, one-tenth the volume of acetic acid is added and the mixture then shaken twice with at least an equal quantity of ether. After washing repeatedly with water, the ether layer is shaken with successive quantities of 5 % HCl and the porphyrin in the combined extracts determined spectrophotometrically (Hilger-Nutting apparatus calibrated by means of standard coproporphyrin solutions, readings being taken at a wave-length of 546–549 mμ in the centre of the β band of the acid absorption spectrum). Frequently, in some pathological urines, the initial acid extract contained brownish pigments without characteristic absorption spectra, which led to erroneously high porphyrin figures, but by careful repetition of the purification over ether and re-extraction with acid [Rimington & Hemmings, 1939] these could usually be eliminated and a reliable result be obtained. Through the kindness of Dr J. E. Kench of the Manchester Royal Infirmary many of the standard analyses were performed in Manchester, whilst the corresponding fluorescence measurements were made at Hampstead. Table 1 presents the results obtained.

ADDENDUM. By E. SCHUSTER

Construction of the apparatus

The apparatus is shown semi-diagrammatically in Figs. 4 and 5 which give respectively a sectional elevation and a sectional plan. It consists of a metal box, the back wall of which (1) is raised upwards for 4 in. and then bent backwards for 2 in. to form a flange (2) which coincides in plan with a backward prolongation of the base (3). Two vertical brass rods (4) connect the base with the flange, and a horizontal bracket (5) is clamped to these rods at an adjustable level. The lampholder (6) for the Osira lamp (7) is screwed to the under-surface of the bracket. Piercing the top of the box near the back wall are two guide tubes, of which the right-hand one receives the test tube (8) containing the standard solution and the left-hand one the sample (9). The bottoms of the test tubes are supported by sockets (10) attached to the base. Ultraviolet rays reach a section of each test tube through windows (11) in the back wall. Small brackets surrounding these windows serve to carry extra pieces of Wood's glass, for use in determinations in which a spectral filter is omitted. They eliminate the last traces of

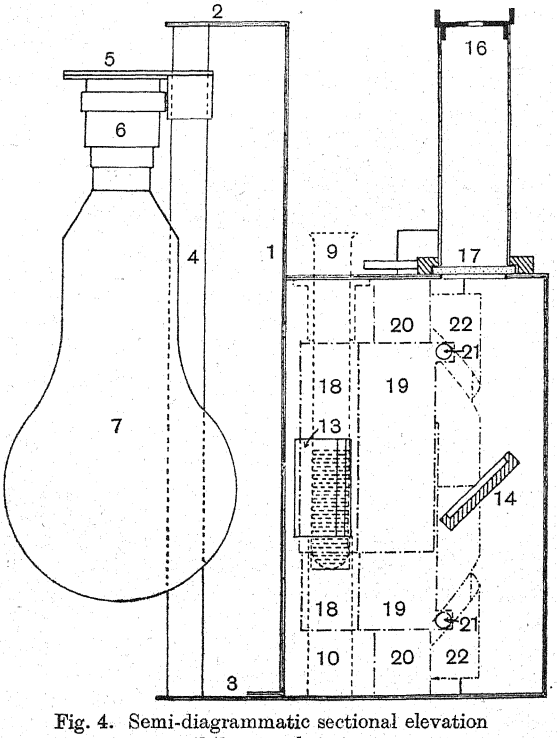

Fig. 4. Semi-diagrammatic sectional elevation (1/3 natural size).

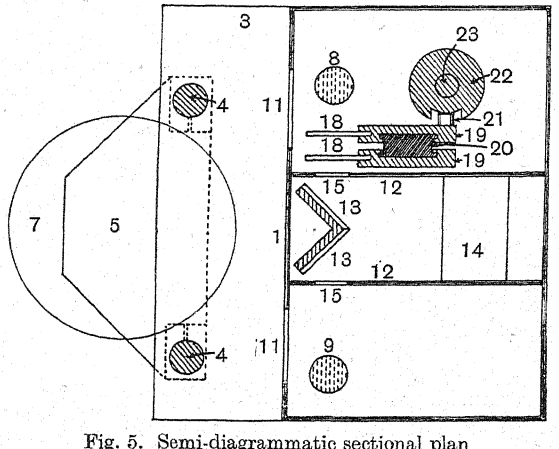

Fig. 5. Semi-diagrammatic sectional plan (1/3 natural size).

visible light from the source. Vertical partitions (12) separate off a central compartment of the box and serve as supports for the three mirrors which it contains. Two of these (13) lie at right angles to each other in vertical planes, while the third (14) makes an angle of 45° with the horizontal.

Fluorescent light from the two test tubes enters the central compartment through square apertures (15); it is reflected by the vertical mirrors on to the oblique mirror and thence upwards into the eyepiece (16) through a hole in the top of the box. The base of the eyepiece tube contains a filter (17) which cuts off practically all light except the wave-lengths produced by the fluorescence of porphyrin.

Interposed between the standard and the aperture (15) are the two glass screens (18); the dark end of one is above and of the other below, so that where they overlap they produce a uniformly shaded field. The screens are mounted in slides (19) which can move up or down a vertical guide (20). Each is held at the desired level by a pin (21) which enters a spiral groove in a vertical cylinder (22). The spiral is in one case right-handed and in the other left-handed. The two cylinders are fixed one above the other to a spindle (23) carried in bearings in the top and base of the box; a knurled head and a dial divided into a hundred divisions are mounted on the spindle above the top of the box. The spiral groove is of such a pitch that the whole range of movement of the slides is achieved by slightly less than one complete revolution of the spindle, and, as the grooves are right- and left-handed, equal and opposite movements are given to the two slides by a given degree of rotation.

The slides are of such a length that their less dense ends are separated by a gap of about $\frac{1}{4}$ in. when the dial knob is at zero, so permitting unobstructed light to pass in this position from the right-hand tube to the mirror for comparison with the tube on the left. This arrangement has been found convenient for certain determinations in which a blank had to be evaluated.

The inside metal work of the apparatus is painted black and the tube of the eyepiece is lined internally with matt surfaced black paper.

Thanks are due to Mr J. Smiles and his assistants of this Institute for kindly preparing the glass screens used in the construction of the instrument. I also wish to acknowledge the benefit of friendly discussions with my colleagues, Dr J. E. Kench of the Royal Infirmary, Manchester and Mr E. M. Jope of the Radcliffe Infirmary, Oxford. Dr Z. A. Leitner kindly secured some of the pathological urines used for test studies.

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The Utilization of Urea in the Bovine Rumen. 1. Methods of Analysis of the Rumen Ingesta and Preliminary Experiments *in vivo*

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Some evidence has accumulated during the past 40 years to suggest that ruminants can utilize simple non-protein nitrogenous compounds such as urea when these compounds replace a proportion of the protein normally present in the diet. The work, much of which is highly controversial, has been reviewed recently by Krebs [1937], and also by Owen [1941]. Since these reviews appeared, publications by Owen, Smith & Wright [1941; 1943] in this country and by Hart, Bohstedt, Deobald & Wegner [1939] in America have shown that urea undoubtedly has some definite nutritive value when fed to ruminants in suitable amounts and in appropriate mixtures. It has been suggested that certain micro-organisms which multiply in the rumen build up their own body protein from this simple form of nitrogen. These micro-organisms then pass further along the alimentary tract where their protein is digested with the ordinary protein of the diet. The work here described was undertaken to test the validity of this theory.

A steer with a large rumen fistula was employed and in the original plans, made in 1938, it was hoped to use this animal, in the first place for *in*

vivo experiments in which urea would be fed and any changes occurring in the N distribution in the rumen ingesta would be observed, and secondly for obtaining samples of the rumen contents for *in vitro* incubations with urea.

It was first necessary to establish reliable methods for the accurate determination of non-protein nitrogen (N.P.N.), urea and NH_3 in rumen ingesta, and also to study the heterogeneous nature of the rumen contents themselves, for it soon became clear that in any experiments carried out *in vivo*, sampling of the rumen contents would present great difficulties. The first paper of this series is therefore concerned with the details of the methods adopted for estimating N.P.N., urea and NH_3, with the heterogeneity of the rumen ingesta of the steer, and with some preliminary *in vivo* experiments.

METHODS

The estimation of N.P.N. in rumen contents

(*a*) *Rumen liquor.* The three protein precipitants which seemed most suitable were trichloroacetic acid, sodium tungstate in H_2SO_4 and alcohol. It is well known that for many substances the estimation of N.P.N. does not result in an absolute value but that different values are obtained by different processes and by slightly different applications of the same process. Tests with rumen liquor were therefore carried out to find how suitable these three precipitants were for the present investigations, and under what conditions they gave reliable values which could be readily duplicated. The sample of liquid rumen contents used contained, after straining through muslin, 2·1% total solids and 80 mg. total N/100 g. The experiments with this sample were planned to study the effect on the N.P.N. value of (1) the concentration of the precipitant, (2) the concentration of the rumen contents in the precipitant, and (3) the time for which precipitation was allowed to continue before filtration.

Precipitation with trichloroacetic acid. Approximately 5 g. of the rumen liquor were accurately weighed into each of a series of flasks and made up to 100 ml. with $CCl_3.COOH$ solution of various concentrations ranging from 2·5 to 20%. The mixtures were thoroughly shaken and then filtered after 24 hr. At the same time further samples of the same rumen liquor were precipitated with 10% $CCl_3.COOH$, but the proportion of the rumen liquor to the total volume of the mixture varied from 1 in 5 to 1 in 50. Again filtration was performed after 24 hr. Finally mixtures were made up consisting of 1 part rumen liquor to 20 parts 10% $CCl_3.COOH$ and portions filtered after 1, 4 and 24 hr. The results are summarized in Table 1.

It appears from these results that with $CCl_3.COOH$ the time which elapses between precipitation and filtration is not of great importance. Up to 4 hr. there was little difference, and after 24 hr. the N.P.N. values had only increased by about 5%. Provided therefore that a definite time be chosen for all determinations, consistent results should be obtained. Decreasing the proportion of rumen contents to $CCl_3.COOH$ solution from 1 in 5 to 1 in 50 increased the N.P.N. value from 24·5 to 28·2, but note that the values obtained for a ratio of 1 in 5 and 1 in 10 differed only very slightly and that the same was true for the two values obtained with ratios of 1 in 20 and 1 in 50. Consistent results should therefore be obtained with this precipitant by keeping the proportion of rumen liquor to $CCl_3.COOH$ approximately constant throughout. The actual concentration of $CCl_3.COOH$ is obviously important, for the higher the concentration the greater is the N.P.N. value. Throughout the present work a 10% solution was used.

Precipitation with sodium tungstate in sulphuric acid. 50 ml. 10% sodium tungstate solution were added to 10 g. of rumen liquor and the mixture made up to 500 ml. with H_2SO_4 of various strengths. It was filtered after 24 hr. The effect of varying the proportion of rumen liquor to precipitant and of varying the interval before filtration was also studied.

Table 1. *Estimation of N.P.N. using trichloroacetic acid as precipitating agent*

(mg./100 g. rumen liquor)

Conc. of trichloroacetic acid (%) (1 part rumen liquor to 20 parts total mixture; filtered after 24 hr.)	N.P.N.	Ratio of rumen liquor to total mixture; made up with 10% trichloroacetic acid (filtered after 24 hr.)	N.P.N.	Time of filtration (hr. after precipitation), 1 part of rumen liquor in 20 parts of total mixture; made up with 10% trichloroacetic acid	N.P.N.
2·5	22·1	1 in 5	24·5	1	25·2
5·0	23·9	1 in 10	25·2	4	25·0
7·0	25·3	1 in 20	27·3	24	26·6
10·0	27·2	1 in 50	28·2		
15·0	33·3				
20·0	35·7				

The results (Table 2) show that when filtration was carried out 24 hr. after precipitation, all concentrations of H_2SO_4 between *N* and *N*/10 tended to give very similar results, whereas with more dilute acid the values were much higher. These high values were probably due to incomplete precipitation, for it was difficult to obtain a clear filtrate at the greater dilutions. The same explanation probably holds for the higher values obtained with *N*/6 acid when filtration occurred 1 or 4 hr. after precipitation. Table 2 also shows that the ratio of rumen liquor to total volume of mixture has little effect on the N.P.N. value provided 24 hr. elapse before filtration.

Precipitation with alcohol. Similar tests were made using alcohol as the precipitant. The results (Table 3) show that the value for N.P.N. increases as the concentration of alcohol diminishes, due undoubtedly to less complete precipitation as the water content of the alcohol increased, since with 96% alcohol a clear filtrate could be obtained almost immediately, whereas with the 60 and 40% alcohols filtration was slow and tended to give turbid filtrates. With 96% alcohol the N.P.N. values were little affected either by the ratio of rumen liquor to alcohol or by the time between precipitation and filtration.

Table 2. *Estimation of N.P.N. using sodium tungstate and H_2SO_4 as precipitating agent*

(mg./100 g. rumen liquor)

Normality of H_2SO_4 (1 part rumen liquor made up to 50 parts total mixture; filtered after 24 hr.)	N.P.N.	Ratio of rumen liquor to total mixture made up with $N/6$ H_2SO_4	N.P.N. filtered after 1 hr.	4 hr.	24 hr.
N	18·8	1 in 5	21·2	20·9	20·5
$N/2$	18·0	1 in 10	21·5	22·2	19·6
$N/6$	17·6	1 in 20	23·6	24·2	18·5
$N/10$	18·2	1 in 50	27·4	27·6	18·0
$N/12$	24·1	1 in 100	33·6	33·1	19·0
$N/16$	24·0				
$N/20$	26·4				

Table 3. *Estimation of N.P.N. using alcohol as a precipitating agent*

(mg./100 g. rumen liquor)

Conc. of alcohol (% by vol.) 1 part of rumen liquor to 20 parts total mixture; filtered after 24 hr.	N.P.N.	Ratio of rumen liquor to total mixture made up with 96% alcohol	N.P.N. filtered after 1 hr.	4 hr.	24 hr.
96	18·3	1 in 5	18·5	18·5	19·2
80	19·5	1 in 10	17·7	18·7	18·3
60	33·1	1 in 20	16·3	16·3	17·7
40	40·3	1 in 50	16·4	17·7	17·6

It is obvious from these first three tables that when filtration was carried out after 24 hr. the 96% alcohol and sodium tungstate methods gave similar results (17·6–19·2 and 18·0–20·5 mg./100 g. respectively), whereas 10% CCl_3.COOH (1 part rumen liquor to 20 parts total mixture) gave much higher values (26·6–27·3 mg./100 g.) although the same sample of rumen liquor was used for all the results recorded in Tables 1–3. Differences of this order (7–10 mg.) between values found by the alcohol and the tungstate methods and the value by CCl_3.COOH have always been observed when the three precipitants have been compared.

Of the three methods investigated the alcohol procedure is the simplest, but it was observed that if phosphate were added to rumen contents as a buffer when urea or NH_3 was present, the alcohol was liable to precipitate a portion of the NH_3, probably as magnesium ammonium phosphate. The alcohol procedure was therefore of very doubtful value as a routine method. CCl_3.COOH was the next choice in order of simplicity. This substance, however, is expensive and large quantities are not readily obtained in war time. Moreover, owing to the inactivation of urease by CCl_3.COOH the estimation of urea in such a filtrate presents difficulties. It was therefore decided to use sodium tungstate and H_2SO_4 as a general routine procedure, confirming the results periodically by parallel tests with CCl_3.COOH.

The method finally adopted was as follows. Approximately 10 ml. rumen liquor, accurately weighed, were added to 50 ml. $N/6$ H_2SO_4 in a 100 ml. flask and the mixture transferred to a 500 ml. volumetric flask with $N/6$ H_2SO_4; 50 ml. 10% sodium tungstate were added and the volume was made up to the mark with $N/6$ H_2SO_4. After standing for at least 24 hr. the mixture was filtered and aliquots of the filtrate were taken for analysis. Various trial experiments showed that the rumen liquor could remain in the 100 ml. flask with H_2SO_4 for at least 2 days before it was made up to 500 ml.; after that, any time between 24 and 48 hr. could elapse before filtration took place without any measurable change in the analytical results.

(*b*) *Solid rumen ingesta.* A sample of the solid rumen ingesta was taken from the rumen at the same time as the sample of the rumen liquor used in the experiments just described. It contained 14·3% of total solids and 290 mg. total N/100 g. The N.P.N. value estimated by 10% CCl_3.COOH was 40·2 mg./100 g., while the corresponding value by the sodium tungstate method was 19·6 and by the 96% alcohol method 20·3. With the solid ingesta therefore, as with the liquor, the alcohol and tungstate methods gave similar results, whereas the figure obtained by using CCl_3.COOH was much higher. Moreover, with the alcohol and tungstate methods the N.P.N. content of the solid ingesta was almost the same as that of the liquid (19–20 mg./100 g.), whereas by the CCl_3.COOH procedure the two values were very different, 40·2 mg./100 g. for the solid and 27·2 for the liquid. The solid and liquid ingesta might be expected to have similar N.P.N. values when estimated on the wet weight, since the solid material, which contains some 86% water, is constantly coming into contact with the liquid and so is likely to be impregnated with it. For this reason, and from these results, the authors believed that in routine work with mixtures of liquid and solid ingesta more accurate and consistent values for N.P.N. would be obtained by the tungstate method than by the use of CCl_3.COOH. The reason for the higher values obtained with CCl_3.COOH is not yet known.

The estimation of ammonia. Aliquots of the filtrate from the tungstate precipitation were neutralized with *N* NaOH using phenol red as indicator. 2 ml. *N* NaOH were then added in excess and the mixture was distilled for 10–15 min. in a current of steam, the distillate being collected in *N*/50 acid. This procedure gave results identical with those obtained when the acid aliquot was rendered alkaline by addition of solid MgO.

The estimation of urea. Aliquots of the tungstate filtrate were neutralized as in the estimation of NH_3, 10 ml. 0·6% KH_2PO_4 were added as a buffer, followed by 5 ml. of a 30% alcohol urease extract [see Peters & Van Slyke, 1932]. The mixture was incubated at 40–45° for 20 min. 2 ml. *N* NaOH were then added and the mixture was distilled in a current of steam for 10–15 min. The distillate was collected in *N*/50 acid.

In both the NH_3 and urea estimations blank determinations were made. The accuracy of the method was tested by determining urea in liquid rumen contents to which urea had been added in varying amounts. The results recorded in Table 4

Table 4. *Estimation of urea using tungstate as precipitating agent*

	(mg. N/100 g.)			
Urea-N added	1·52	3·04	6·08	7·60
Urea-N found	1·44	2·93	6·13	7·54
Recovery (%)	95	96	101	99
Urea-N added	15·2	30·4	60·8	152·0
Urea-N found	15·7	30·2	60·2	151·0
Recovery (%)	103	99	99	99

show that with less than 6 mg. urea-N/100 g. rumen contents the accuracy is about 95%, but that above that value the accuracy closely approaches 100%.

The heterogeneous nature of the rumen ingesta

The bovine rumen is an enlargement of the oesophagus and may contain as much as 200 lb. of material. The upper portions of the ingesta have a consistency similar to that of horse faeces, but as the rumen is descended the mass becomes increasingly moist, until 12–18 in. below the fistula the solid contents are bathed in fluid. At frequent intervals the whole mass of liquid and solid is mixed together by movements of the rumen musculatory system. A sample truly representative of this heterogeneous mass at any given time is difficult to obtain. Determinations of N distribution values on samples taken at the same time from different positions in the rumen showed a variation between extremes of almost 20%, and marked differences were also observed when samples of the material were taken from different depths in the rumen. In Table 5, the total N and N.P.N. values are recorded for moist but solid samples taken from different levels of the rumen. The N.P.N. values in particular show very wide variations. At depths below 8–12 in. the sampling difficulties become greater, for at these levels the proportion of liquid to solid withdrawn can be varied at will by the sampler. The sampling difficulties apparent in the results recorded in Table 5 are therefore much greater for samples taken from lower levels in the rumen where (below 8 in.) the greater part of the rumen contents is to be found.

Table 5. *Concentration of total nitrogen and N.P.N. of rumen ingesta from different depths of the rumen*

Depth below surface of ingesta in.	Water %	Fresh rumen contents (mg./100 g.)		Dry matter (mg./100 g.)	
		Total N	N.P.N.	Total N	N.P.N.
1	82·5	342	15·8	1949	90
4	84·3	321	20·1	2044	128
6	85·1	307	11·9	2053	80
8	83·9	288	12·3	1788	77
% difference between extremes	3·1	15·8	40·8	12·9	39·8

One of the aims of these experiments was to feed urea in a typical basal diet and study the changes in the rumen ingesta at intervals after the meal. Preliminary tests were therefore made to detect any variations in the total N of the rumen ingesta throughout the 10 hr. following a urea-free meal. Each sample analysed was a mixture of samples taken from four different parts of the rumen (see Table 6). It will be seen that total N values varied

Table 6. *Nitrogen content of rumen ingesta at hourly intervals after meal*

Time after meal hr.	Moisture %	Total N (mg./100 g.)	
		Fresh rumen contents	Dry matter
1	80·0	360	1800
2	80·2	322	1628
3	80·5	362	1856
4	82·4	302	1716
5	82·0	311	1728
6	81·9	316	1746
7	81·6	322	1750
8	81·7	360	1967
9	81·9	328	1817
10	82·0	312	1733

very irregularly from 311 to 362 mg./100 g. for wet rumen contents, and from 1628 to 1967 mg./100 g. for dry matter. The authors believe that these variations were due mainly to sampling difficulties which made it impossible to detect with certainty the conversion of urea to protein in the rumen after a urea meal unless the amount of conversion were much greater than could be expected.

It was originally intended that extensive *in vivo* experiments should be done. The N distribution in the rumen on a diet containing no added urea was to have been determined. Urea was then to be included and its conversion into protein closely observed. After preliminary experiments, however, it appeared that sampling difficulties made precise

information as to the N distribution throughout the whole of the rumen ingesta at any given time unobtainable. Without such information any attempt to follow the conversion of urea to protein in the intact rumen was of little value.

Another difficulty also arises in interpreting the results obtained in *in vivo* experiments, because portions of the rumen contents are periodically passing from the rumen and reticulum into the other stomachs, and the material passing on at any time may not be representative of the rumen contents as a whole. It follows that if the small amount of urea or ammonia N present in the rumen immediately after a urea meal decreased within the next few hours, the decrease might be partly due to the passing of water-soluble urea or NH_3 into the other stomachs and intestines, and not necessarily to their conversion to protein. Even a simultaneous increase in the amount of total N in the dry matter of the rumen contents might be due to a selective passing-on of water-soluble carbohydrates and other constituents of the diet. These difficulties are illustrated by the results of an experiment in the *in vivo* section of the general investigations.

For 6 months the steer had received daily a mixture consisting of 2¾ lb. oats, 2 lb. bran, 1 lb. starch, ⅔ lb. molasses, 40 g. urea, 14 lb. hay and water *ad lib.* During this particular experiment the concentrate mixture was given in two portions, one at 9.45 a.m., the other at 4.30 p.m. Samples of rumen contents were removed at 9.30 a.m., 10.30 a.m., 12.30 p.m. and 3.30 p.m. The 9.30 samples should therefore have been representative of the rumen ingesta after a fast of 17 hr., the others of the material at varying periods immediately after the meal. At each sampling period semi-solid samples of approximately 600 g. were collected from near the top at each of the four 'corners' of the rumen and a fifth more liquid sample from deep down in the centre of the rumen. Without further treatment these five samples were analysed immediately in duplicate for water, total N and N.P.N. contents. Three days and also 6 days later this procedure was repeated. Thus, while the steer was receiving the basic diet plus urea, 96 values were obtained for the content of both the total N and the N.P.N. in the semi-solid ingesta, and 24 values for both these constituents in the liquid ingesta. Urea was then omitted from the basic diet and 9, 12 and 15 days later, the sampling and analytical procedure were repeated. The detailed results are too numerous to publish in their entirety. It appeared, however, that with both the semi-solid and the more liquid samples duplicate determinations for the same sample frequently differed by 20% or more. A similar difference was often observed in the values obtained for samples taken at the same time but from different parts of the rumen. Such large divergencies in individual values were too frequent to permit conclusions to be drawn as to the synthesis of protein from urea in the rumen.

The average results (Table 7) gave no evidence either for or against the theory that urea is utilized in this way. A study of Table 7 shows the following points:

(1) The concentrations of crude protein, true protein and N.P.N. were higher in the dry matter of the liquid ingesta than in that of the semi-solid material. In any quantitative experiment therefore the proportion of solid to liquid ingesta, and in fact the total amounts of each in the rumen, would have to be known.

(2) There was no significant difference between the protein contents of the rumen ingesta when the diet was fed alone and when it included urea.

(3) The only significant changes on the urea diet were in the N.P.N. fraction, e.g. 144 mg./100 g. in the dry matter of the liquid ingesta just before a meal had increased to 313 mg./100 g. 30 min. after the meal and had fallen again to 119 mg./100 g. some 5½ hr. later; the corresponding values for the semi-solid ingesta were 74, 118 and 59 mg. N/100 g.

Table 7. *Three-day average values for the content of crude protein, true protein and N.P.N. in the rumen ingesta on a diet containing urea and on the same diet without urea*

Time after previous meal hr.	Crude protein % dry matter		True protein % dry matter		N.P.N. mg. N/100 g. dry matter	
	Urea	No urea	Urea	No urea	Urea	No urea
	Semi-solid ingesta					
17	11·6	10·6	11·1	10·2	74	62
0·5	10·1	10·3	9·4	9·9	118	68
3	10·2	10·1	9·7	9·8	83	44
6	11·0	10·7	10·6	10·3	59	58
	Liquid ingesta					
17	15·1	17·2	14·2	16·7	144	83
0·5	17·3	15·6	15·3	14·8	313	130
3	16·3	14·9	14·6	14·1	276	128
6	15·3	15·8	14·6	14·9	119	139
Average of 96 duplicate determinations involving 48 samples of semi-solid ingesta	10·7	10·4	10·2	10·0	84	58
Average of 24 determinations involving 12 samples of liquid ingesta	16·0	15·9	14·7	15·1	213	120

Crude protein = 6·25 × total N; true protein = 6·25 × (total N − N.P.N.).

To show that urea was converted to protein in the rumen such an experiment should yield results such that the decrease in N.P.N. following the urea meal could be correlated with an increase in the 'true protein'. But here, if the fall in N.P.N. were entirely due to protein synthesis, a decrease in N.P.N. from 118 to 59 mg. N/100 g. and from 313 to 119 would mean an increase in true protein of only 0·4 and 1·2 % respectively. The value of 9·3 % for the true protein of the semi-solid ingesta 30 min. after the meal (Table 7) would therefore have to increase by 0·4–9·7 %, some hours later, an increase which would not be significant owing to sampling errors. Similarly, the value of 15·3 % for the liquid ingesta would have to increase to 16·7 %. Actually in the solid material the value rose to 9·7 % after 3 hr. and 10·6 % after 6 hr., but in the liquid ingesta it decreased to 14·5 %. These results therefore supply no proof of protein synthesis from urea. They are, however, not at variance with the suggestion that protein synthesis from urea did take place, because throughout the day the animal continued to drink at intervals and to secrete the usual large quantities of saliva. Moreover, part of the ingesta, particularly in the more liquid form, would be continually passing from the rumen. These processes would all tend to induce a diminution in protein and in N.P.N. content of the liquid ingesta as the interval after the meal increased. Simultaneously the semi-solid rumen ingesta might become slightly richer in protein because the more soluble carbohydrate material was washed out of the animal's food. By increasing the urea in the diet during the urea period the problem would not be solved but made more difficult, as the greater urea consumption would increase the water ingested and this would enhance the protein content of the solid ingesta and decrease that of the liquid.

DISCUSSION

The detection of urea conversion to protein by an *in vivo* experiment of this type is extremely difficult and seems never likely to yield results completely beyond dispute. Thus it seemed wise, at least in the early stages of the work, to concentrate on incubation experiments carried out *in vitro* when these difficulties would not arise, and to postpone the *in vivo* section of the general investigations for the time being. The main portion of the work completed therefore consists almost entirely of the *in vitro* experiments to be discussed in the next two papers of this series.

Since the original *in vivo* programme was temporarily abandoned, Wegner, Booth, Bohstedt & Hart [1941] have published a description of *in vivo* experiments using a heifer with a rumen fistula. The values for N.P.N. and total N of the rumen contents at intervals after the ingestion of a basal diet were compared with the corresponding values obtained when the diet included urea. Wegner *et al.* found that in general the crude protein content of the rumen ingesta was higher with added urea than it was on the basal diet alone, and that the N.P.N. of the ingesta fell to the basal level during the first few hours after a meal. They therefore suggested that urea utilization takes place in the rumen and that it occurs within 4–6 hr. after ingestion. An examination of the results, however, shows that such an interpretation is not necessarily correct, and that these workers have evidently encountered those very difficulties which the present authors anticipated. Thus from their 1st experiment it can be calculated that on the basal diet alone the true protein of the rumen ingesta appeared to increase from 8·4 % at 1 hr. after a meal to 11·3 % 4 hr. later, a difference of 2·9 %. This was accompanied by a decrease in N.P.N. equivalent to only 1·0 % true protein. When, however, the diet included urea, the corresponding increase in true protein was only 0·9 % (9·9–10·8 %), and this was accompanied by a decrease in N.P.N. equivalent to 1·5 % protein. There therefore appears to have been less synthesis of protein with urea than with the basal diet alone during the 5 hr. following the meal. Again, the value of 11·3 % for the true protein 5 hr. after the basal diet was fed, was actually slightly higher than 10·8 %, the corresponding value when urea was included in the diet, a condition reversed 4 hr. later without any significant change in N.P.N.

In their 2nd experiment Wegner *et al.* [1941] fed a very abnormal basal diet consisting of 15 lb. corn silage and 3 lb. starch, following this by a period on the same basal diet with the addition of urea amounting to 5 % of the dry matter of the ration. They found that of several diets which they tested, this most unusual one, which must have contained only about 5 lb. dry matter, gave the most marked results. Their values show that on two of the days when the basal ration was fed alone, the crude protein content of the rumen ingesta increased by 0·45 and 0·85 % between either 1 or 2 and 8 hr. after the meal, but that on the only other day for which the corresponding values are available it decreased by 1·7 %. No conclusion can be reached therefore as to whether the crude protein content of the rumen ingesta should be expected to rise or fall during the first 8 hr. after the ingestion of the basal diet. On the 2 days for which the corresponding values are given for the basal diet + urea, the crude protein decreased by 0·7 % between 2 and 8 hr. after feeding on the first day, and remained unchanged on the second. Even correcting the crude protein for NH_3 where possible (N.P.N. values are not given), the maximum increase in protein following the ingestion of urea was only 0·6 %. In relation to the changes occurring on the

basal diet alone, the results do not support the view that urea is converted to protein in the rumen during the first 8 hr. following a meal.

The evidence upon which Wegner *et al.* [1941] appear to place most confidence is that on the basal diet alone the crude protein content of the rumen ingesta varied from 8·9 to 10·6 %, whereas with the addition of urea to the diet this value became much higher and varied between 12·0 and 12·7 %. In all probability, however, these values might be due to the fact that when 100 g. urea were added to the diet which supplied only 5 or 6 lb. dry matter per day, of which 3 lb. was starch, water consumption and also salivation would be much increased. This would cause the starch and the more soluble matter in the corn silage to be washed from the rumen much more rapidly than in the absence of urea. The proportion, in the dry matter of the rumen ingesta, of fibre and of the less soluble protein enmeshed in it would thus be increased.

These experiments of Wegner *et al.* [1941] therefore confirm the views held by the present authors that *in vivo* experiments of this type cannot be expected to yield any certain evidence of protein synthesis from urea until truly representative samples of the total ingesta can be obtained and analysed, and until more is known of the effect of urea on the passage of the various dietary constituents through the rumen.

SUMMARY

1. The relative merits of trichloroacetic acid, sodium tungstate with H_2SO_4, and alcohol have been compared as precipitants in the estimation of N.P.N. in rumen ingesta.

2. A description is given of the methods finally adopted for the estimation of N.P.N., urea and NH_3.

3. The heterogeneous nature of the rumen contents is described and illustrated by analyses.

4. The difficulties involved in the interpretation of the results obtained by *in vivo* experiments are discussed.

5. Owing to these difficulties, the results of *in vivo* experiments of this type cannot be regarded as supplying evidence either for or against the theory that urea is converted to protein in the rumen.

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The Utilization of Urea in the Bovine Rumen. 2. The Conversion of Urea to Ammonia

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In the previous paper of this series [Pearson & Smith, 1943] evidence was advanced to show that the interpretation of experiments carried out *in vivo* on the utilization of N.P.N. by ruminants was so difficult as to render the work of little value and that more reliable results could probably be obtained by *in vitro* methods. A plan of investigation was therefore prepared in which samples of rumen ingesta were to be incubated in the laboratory with and without urea, and the N partition studied at intervals throughout the incubation period. At the outset of the work it appeared that even when 40 g. urea were fed per day to a steer with a rumen fistula, no urea could be detected in the rumen ingesta. In preliminary incubation experiments it was also found that 100 g. rumen contents were able to convert some 100 mg. urea to NH_3 in 1 hr. It is probable therefore that any utilization of urea by ruminants involves the conversion of urea to NH_3 as the first stage of the process. A detailed study of this conversion has therefore been made, with particular reference to the effect upon it of various factors such as temperature, *p*H, concentration of urea, nature of the gases present during incubation and certain inhibitory substances. The present paper consists of a brief description of these experiments.

METHODS

Samples of rumen contents were obtained as follows. 2–3 l. semi-solid rumen contents were transferred with an aluminium cup by way of the fistula to a large flask contained in a bucket of sawdust to minimize heat losses. The sample was strained through muslin and the residue pressed by hand. The 'filtrate' so obtained was rich in Protozoa and bacteria and was very similar to the rumen contents as a whole except that the small amount of solid vegetable matter which it contained was in a more finely divided form. The greater part of the solid ingesta of the rumen and reticulum is impregnated with this liquid which, in converting urea to NH_3 and NH_3 to protein, probably behaves very similarly to the more solid ingesta. Since no sampling difficulties arise with this liquid it was used throughout the present experiments and is referred to as the 'rumen liquor'.

Incubation of the rumen liquor was carried out in a water-bath thermostatically controlled at 39°, the body temperature of the bovine. Throughout the greater part of the experimental period the diet of the steer from which the samples were obtained consisted of 14 lb. hay, $2\frac{3}{4}$ lb. oats, 2 lb. bran, 1 lb. starch, 0·7 lb. molasses and 40 g. urea per day with water *ad lib.* The methods of analysis have already been described [Pearson & Smith, 1943].

RESULTS

Influence of CO_2 and N_2 on incubation. One of the objects of the present experiments was to imitate as far as possible in the laboratory the conditions in the intact rumen. In the rumen there are large amounts of carbohydrates such as cellulose, starch, and their breakdown products, and the gas formed in the decomposition of some of these substances usually contains about 62 % CO_2. A few experiments were therefore made to discover whether the nature of the gas and the presence or absence of starch affected the urea-splitting power of the rumen contents. Three 650 ml. aliquots from the same sample were incubated with 100 ml. of a solution containing 1 g. urea, 4 g. K_2HPO_4 and 10 g. glucose. A constant stream of CO_2 was passed through the 1st flask, air through the 2nd and N_2 through the 3rd. Urea was estimated every 10 min. for 2 hr. The results illustrated in Fig. 1 show that the rate of urea conversion was much the same with all three gases. It appeared, however, in the final stages to be slightly more efficient with CO_2 than with air or N_2.

In a 2nd experiment of this type with a much less active rumen liquor, aliquots of the same sample were incubated with air and CO_2, both with and without starch. KH_2PO_4 was present as a buffer but glucose was not included. The results shown in Fig. 2 suggest that CO_2 in the presence of starch accelerates the rate of conversion of urea slightly; otherwise no very significant differences were observed.

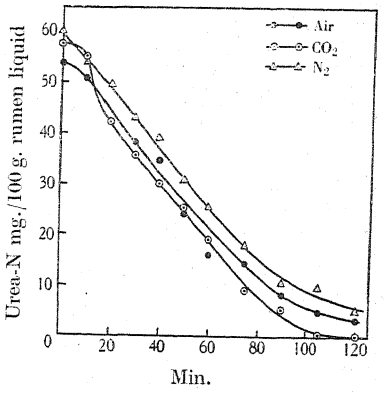

Fig. 1. The conversion of urea in the presence of different gases.

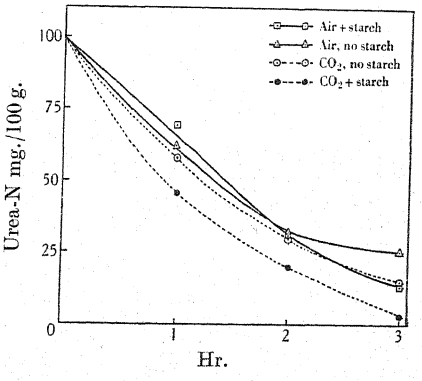

Fig. 2. The effect of CO_2 and starch on the rate of urea conversion.

The effect of starch and phosphate was also tested by incubating them with two different samples of rumen contents in air under strictly comparable conditions. The results are recorded in Table 1.

Table 1. *Ammonia-N produced from urea in 1 hr. at 39°*

(mg./100 g.)

Substances added	Sample 1	Sample 2
Urea alone	27·1	36·7
Starch + urea	31·2	36·5
Starch + urea + phosphate	21·5	30·1
Phosphate + urea	—	30·1

Starch appeared to have a slight accelerating action on the rate of urea conversion with the 1st sample but no effect whatever with the 2nd, which

was the more active of the two. Phosphate obviously had a retarding effect with both samples.

Influence of temperature. 5 ml. of a solution containing 3 % KH_2PO_4 and 3 % starch were placed in each of ten 200 ml. flasks which were kept in water baths at temperatures ranging from 4 to 89°.

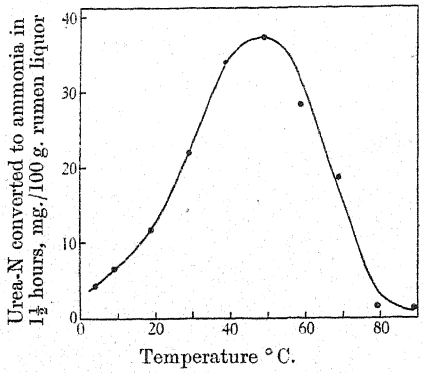

Fig. 3. The effect of temperature on the rate of conversion of urea to ammonia by the rumen liquor.

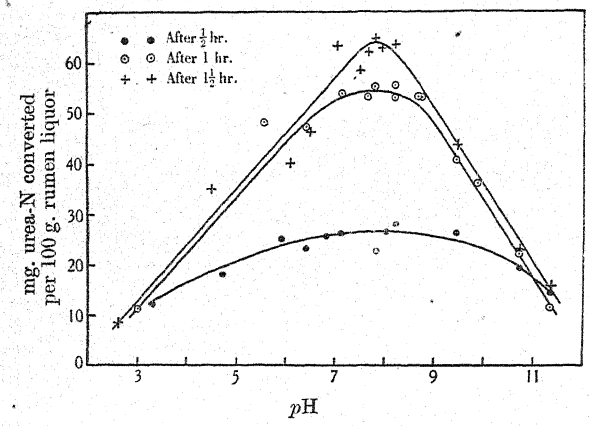

Fig. 4. The effect of *p*H on the urease activity of the rumen contents.

A 50 ml. aliquot from one sample of rumen contents was added to each flask and the contents of the flask were allowed 10 min. in which to reach the temperature of the surrounding bath. Exactly 5 ml. of a solution containing 1 g. urea-N/100 ml. were then added to each flask, and the mixture was incubated for 2 hr. At 10-min. intervals a slow stream of CO_2 was passed through the contents in each flask. At the end of the incubation, urea and NH_3 were estimated. KH_2PO_4 was added in this instance to minimize any loss of NH_3 which might occur at the higher temperatures. The results (Fig. 3) show that urea conversion is insignificant at 4°, but that it increases with increase in temperature and reaches a maximum at 49°. Thereafter it steadily decreases till at 79° there is almost complete inactivity. In two other incubations lasting 1½ and 2 hr. respectively with entirely different samples of rumen liquor, in which no starch was present and through which no CO_2 was passed, the optimum temperature was again 49°.

Influence of p*H.* The technique was similar to that used in the above experiments but the temperature remained constant at 39° while the *p*H in the various flasks ranged from 3 to 11. Three series of incubations were carried out, one for 30 min., another for 1 hr. and a third for 1½ hr. It is probable that during the longer incubations, the salts in the buffer used to obtain a certain *p*H had a more marked effect in retarding urea conversion than the *p*H itself. This may be the cause of anomalous results (see Fig. 4), particularly on the acid side of neutrality. The urease activity of the rumen liquor appears to be very marked over quite a large *p*H range. The optimum probably lies between 7 and 9 and there is little activity below 3 or above 9·5.

The *p*H of the material in the rumen probably varies slightly with a number of factors, but it is rarely below 6·5, and only after a long fast does it approach 7·4. Acid products formed in the rumen are partially neutralized by the incoming saliva, but the buffering capacity of the rumen liquor itself is very high, particularly in view of the fact that its total content of solids is only 2–3 %. This is illustrated by the fact that when 5 ml. of approximately *N* HCl were added to 50 ml. rumen liquor the *p*H was 4·7, whereas when 50 ml. of water were used instead of rumen liquor the corresponding value was 1·6.

Influence of urea concentration. In some enzymic reactions the concentration of the substrate has a marked effect on the enzyme activity. To ascertain whether the concentration of urea influenced the urea-converting activity of rumen liquor, various aliquots of the same sample of ingesta were incubated for 1 hr. with different amounts of urea and the liberated NH_3 was estimated. The NH_3 in the tungstate filtrate was not determined by distillation in steam, for with higher concentrations of urea small but significant amounts were converted to NH_3 during the actual

Table 2. *The amount of urea converted to ammonia in varying concentrations of urea*

	(mg. N/100 g.)				
Urea-N in the rumen liquor	30·8	50·0	116	216	414
NH_3-N produced in 1 hr.	17·9	19·2	20·1	20·6	22·2
Urea-N in the rumen liquor	808	1302	1646	3330	
NH_3-N produced in 1 hr.	24·8	25·1	25·4	25·1	

distillation, however accurately the filtrate was neutralized. Prolonged aeration at room temperature in the presence of K_2CO_3 was therefore adopted. The results recorded in Table 2 show that there was a slight increase in the conversion of urea with increased substrate concentration. The effect, however, was not great.

The influence of time of sampling. When urea-feeding is adopted the question arises whether it should be given twice a day with the other concentrates or ingested at more frequent intervals. The following experiments were therefore made to see whether the urea-converting activity of the rumen liquor varied throughout the day.

A sample of rumen liquor removed 1 hr. after a meal was found to convert urea to NH_3 at the rate of 13·4 mg. N/100 g. rumen liquor in 20 min. After a 16 hr. fast the corresponding value was 14·8 mg. There was therefore no significant difference. In a 2nd test a sample taken 17 hr. after the previous meal converted 27·6 mg. urea-N to NH_3 in 1 hr. A meal was then given and samples taken at 2-hourly intervals for 8 hr. During this time the conversion rate remained unchanged at 23·4–23·8 mg. N/100 g. In a 3rd test the value 17 hr. after a meal was 46·3 mg. urea-N/100 g./hr. Samples taken during the next meal and subsequently at hourly intervals gave conversion rates which varied irregularly between 35·4 and 52·3. If the weight of the rumen contents be estimated at 75 kg. these results mean that 40–80 g. of urea could be converted to NH_3 in the rumen in 1 hr. It therefore follows that during urea-feeding, when as much as 100 or 120 g. urea may be ingested in one day, it matters little so far as the urea conversion to NH_3 is concerned whether this be given twice a day in two equal amounts with other concentrates or at more frequent intervals.

Rumen urease

Since the constituents of the diet of the steer during these experiments showed only insignificant amounts of urease activity, and since there is no active enzyme secretion into the rumen except the saliva, the rumen urease must be generated by some of the micro-organisms present in the paunch. From the amounts of urea which can be converted by the rumen liquor in 1 hr. or even in a few minutes at any time throughout the day, it may be assumed that the enzyme is generated in large amounts compared with the rate of growth of the bacteria producing it. Preliminary attempts were made to prepare a sterile but active filtrate of rumen liquor. The filtrate from Berkfeld filters was sterile but without urease activity. With urease extract from jack bean meal also Berkfeld filtration removed the greater part of the urease activity. Since the molecular weight of crystalline urease from soya beans has been found by Sumner, Gralen & Eriksson-Quensel [1938] to be 483,000 it is not surprising that in the present work no filter has been found which will allow the enzyme to pass without the bacteria.

An attempt was made to concentrate the urease activity, and to effect at least a partial separation of the enzyme from the bacteria, by centrifuging. 2 l. of rumen contents were centrifuged for 5 min. at 1000 r.p.m. and 200 ml. of the top portions of the supernatant liquid collected. 200 ml. of the sludge at the bottom of the centrifuge cups, which was extremely rich in Protozoa and bacteria, were also collected. The top layers, the sludge and also some of the original rumen contents which had been centrifuged and then remixed, were each incubated with about 260 mg. urea, and samples were analysed every 30 min. for 2½ hr. The results shown in Fig. 5

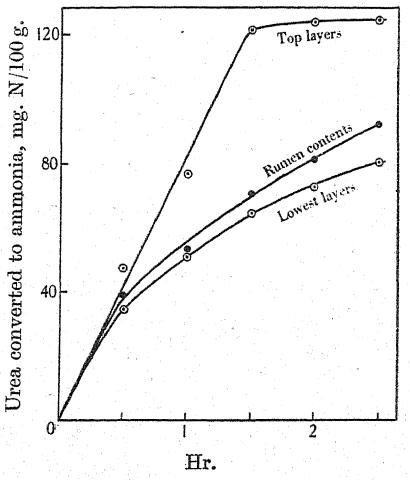

Fig. 5. The rate of urea conversion in the rumen liquor compared with that in the top and bottom layers of the centrifuged rumen liquor.

suggest that for the first ½ hr. or, until some 40 mg. urea-N had been converted to NH_3, the rate of conversion in the lower layers was slightly less than in the rumen liquor, while in the top layers it was slightly greater. At the end of 1 and 1½ hr. the increased rate in the top layers was still more apparent compared with that in the other two. In other experiments in which very much less urea-N was present, and in which conversion was complete in 30–60 min., this difference was by no means clear. The less rapid rate in the rumen liquor and lower layers in the first experiment may have been due less to a smaller content of urease than to a greater concentration of inhibitory substances capable of reducing their urease activity, particularly after the first ½ hr. The results shown in Fig. 5 therefore need not indicate a concentration of urease in the upper layers on centrifuging.

Toluene, which is known sometimes to destroy bacteria but to permit enzyme action, was found to be without effect on the urease activity of the rumen liquor, but here it is not certain whether the urea-converting bacteria were completely destroyed by that treatment. Reference will be made to this in a subsequent paper. 30 % alcohol extracts of the urease were inactive.

Urease activity was also inhibited by small quantities of substances such as quinone and NaF. Quastel [1933] has shown that the activity of ordinary urease preparations is readily inhibited by quinone, and that the inhibition can be prevented by thiol compounds such as H_2S and cysteine. Samples of rumen liquor containing urea were therefore incubated for 1 hr. with varying amounts of quinone and with quinone in the presence of H_2S and cysteine (Tables 3 and 4). With one sample of rumen liquor concentrations of quinone as low as 5 in 100,000 substantially reduced the urease activity. At higher concentrations the degree of inhibition increased and ultimately approached completion at 3 parts in 10,000. With sample 1 H_2S neutralized the inhibitory action of quinone up to concentrations of 0·01 %. With sample 2 the neutralization of inhibition was marked but not quite complete. Cysteine at concentrations of 0·01 % or above also completely neutralized the inhibitory action of 0·01 % quinone (Table 4). Cysteine itself was found to have a very slight inhibitory effect.

Table 3. *The inhibitory effect of quinone on rumen urease and its neutralization by H_2S*

Urea-N converted to NH_3 (mg.)

Quinone %	Sample 1		Sample 2	
	Quinone	Quinone + H_2S	Quinone	Quinone + H_2S
0·0	71·1	71·4	61·2	58·6
0·005	26·8	69·3	13·1	50·9
0·0075	14·2	69·7	2·4	49·9
0·01	13·2	68·2	1·0	33·5
0·03	5·0	10·4	—	—
0·05	3·0	4·3	—	—

Table 4. *The influence of cysteine on the urease-inhibiting action of quinone*

0·01 % quinone + cysteine (%)	0·0	0·005	0·01	0·03
Urea-N converted to NH_3 in 1 hr. (mg.)	1·6	25·2	64·4	67·5
0·01 % quinone + cysteine (%)	0·05	0·07	0·10	
Urea-N converted to NH_3 in 1 hr. (mg.)	64·4	66·1	62·6	

0·05 % cysteine in the absence of quinone gave a value of 63·5.

Thus 0·05 % cysteine in rumen liquor reduced the rate of urea conversion from 47 to 43 mg. N/100 g. rumen liquor/hr. These results for urease activity of rumen liquor are similar to those of Quastel [1933] for urease from jack bean meal, and show that the enzymes from these two very different sources have much in common and may be identical.

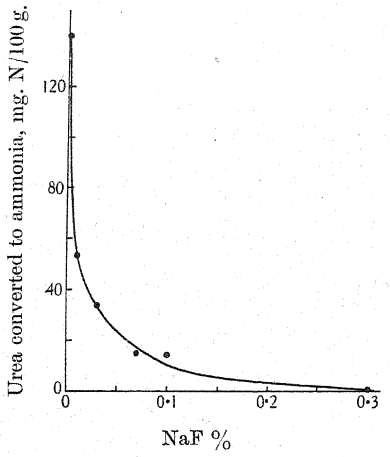

Fig. 6. The inhibition of urease activity in the rumen liquor by sodium fluoride.

Mystkowski [1928] has shown that NaF is a powerful inhibitor of ordinary urease. This was found to be true also for the urease of the rumen. Aliquots of a sample of rumen liquor containing urea were incubated for 2 hr. with various concentrations of NaF. At a concentration of 0·01 % the activity was reduced by about 50 % and at 0·3 % inhibition was complete (Fig. 6). This effect is due entirely to the fluoride radical, for in a control experiment with 0·5 % NaCl there was no inhibition. A similar experiment was carried out with boric acid and borax (Table 5). 0·05 % borax had no inhibitory effect and may even have stimulated the urease action slightly. Above this concentration, however, the amount of urea conversion was dimin-

Table 5. *The inhibition of urease activity by boric acid and borax. Urea conversion to NH_3*

(mg. N/100 g.)

Boric acid or borax %	Boric acid		Borax	
	30 min.	60 min.	30 min.	60 min.
None	28·5	46·8	28·5	46·8
0·05	21·7	40·6	28·3	48·2
0·10	19·5	36·5	24·3	43·3
0·50	11·7	22·3	13·0	25·0

With further samples of rumen liquor 1 and 2 % boric acid and borax completely inhibited the urease activity.

ished significantly. At the lower concentrations inhibition with boric acid was greater than with borax.

SUMMARY

1. In the utilization of urea by dairy cattle the first stage is probably the conversion of urea to NH_3 in the rumen. Moreover, the urease activity of the rumen ingesta is so great at all times of the day, whatever the time of feeding, and remains so little affected by relatively large amounts of urea, that all the urea ever likely to be fed, even to a high yielding cow in full lactation, would readily be converted to NH_3 within 1 hr. This fact has been confirmed by Wegner, Booth, Bohstedt & Hart [1941], who found a marked increase in N.P.N. and NH_3 in the rumen after a urea meal but were unable to detect urea itself.

2. The urease of the rumen resembles, in activity, that from soya and jack beans. Changes in its activity with changes in temperature and *p*H, and its behaviour in the presence of such inhibitors as quinone and NaF, are typical of enzymes of the urease type. Preliminary attempts to obtain enzyme preparations free from bacteria have, however, proved unsuccessful.

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The Utilization of Urea in the Bovine Rumen. 3. The Synthesis and Breakdown of Protein in Rumen Ingesta

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The urease activity of bovine rumen ingesta has been studied in detail by Pearson & Smith [1943*b*] who showed that the urease activity of the rumen is sufficient to convert rapidly to NH_3 all the urea ever likely to be included in the diet as a partial substitute for protein. The utilization of urea by the ruminant probably takes place in two stages, first the conversion of urea to NH_3 and second the conversion of NH_3 to protein. The present section of the work was undertaken to investigate this 2nd stage. Incubations of rumen contents were to be carried out *in vitro* under a variety of different conditions with and without urea, to obtain as much evidence as possible by chemical analysis either for or against the theory of protein synthesis in the rumen from non-protein N. Samples were to be taken for direct microbiological examination by Mr Frank Baker [Baker, 1942 *a*, *b*; 1943] and for plate counts by Dr C. Higginbottom to discover whether the protein synthesis, if it occurred, were accompanied by an increase in the numbers of micro-organisms present.

First, it had to be established by chemical means whether any protein synthesis could be detected, and if so, to what extent it was affected by the presence of ordinary dietary constituents such as carbohydrates, proteins and amino-acids, as well as by substances which might be of a toxic nature. The present paper is confined to a description of this particular aspect of the work.

METHODS

The method of obtaining samples of rumen liquor and the incubation and analytical procedures have already been described [Pearson & Smith, 1943 *a*, *b*].

Long-period incubations. It seemed possible that if any measurable change in N partition took place on incubating rumen contents *in vitro*, the changes would be more readily detected in fairly long incubations. Hence preliminary incubations with rumen contents were carried out, each lasting several days (Fig. 1). 900 ml. rumen liquor were incubated with 2·5 g. urea, 12 g. K_2HPO_4, 10 g. glucose and 0·1 g. $FeSO_4$ for 8 days. Note that while the total N remained constant throughout, the N.P.N. varied considerably. For the first 3–4 days it changed very slightly, but between the 3rd and 7th days it greatly decreased. Assuming that the difference between total N and N.P.N. is protein N, an assumption which is discussed later, Fig. 1 shows that the amount of protein in the medium changed little at first, but that during the last 5 days it increased

substantially. The explanation may be that many micro-organisms in the rumen ingesta ceased to multiply on removal from the rumen and incubation, but that after 3–4 days the strains which had survived and for which the stagnant rumen contents were a suitable medium passed from a stationary phase to a phase of active growth in which rapid multiplication occurred. Several experiments of this type were performed with and without urea, and although the results varied in detail, the general trend of the curves obtained was always the same.

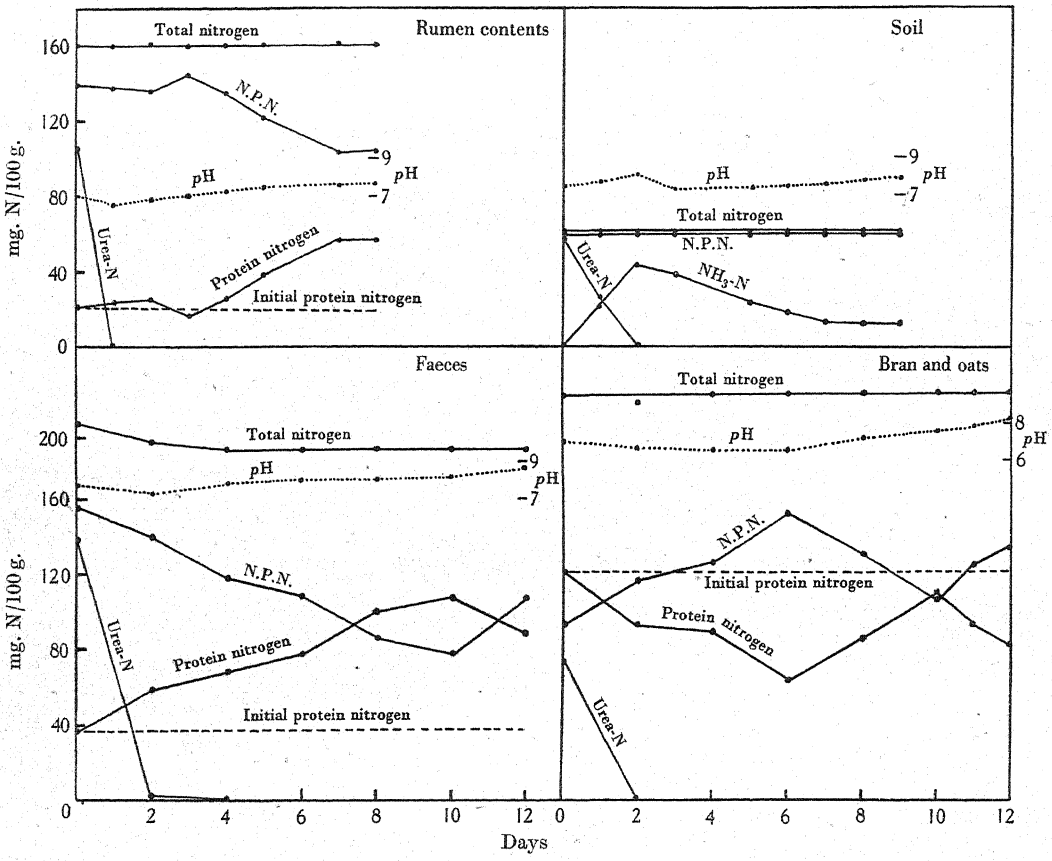

Fig. 1. The results obtained from prolonged incubations of rumen contents and also from prolonged incubations of suspensions of faeces, feeding-stuffs and soil. The amount of protein synthesis with rumen contents and faeces can be found by comparing the initial protein nitrogen with the protein nitrogen observed at different times throughout the incubation period. (In order to make the diagrams as simple as possible NH_3-N is not shown for rumen contents, feeding-stuffs and faeces. In all three instances after urea was converted to ammonia, the NH_3-N followed a course parallel to that of the N.P.N.)

To ascertain whether these changes in N.P.N. were associated with the presence of micro-organisms in the rumen contents or were fortuitous changes inherent in the experimental procedure, Mr Frank Baker suggested that parallel experiments be done with bovine faeces, with soil suspensions and also with suspensions of bran and oats in which the bacterial content would be widely different from that of rumen liquor. Bran and oats were chosen because they formed an appreciable part of the diet of the steer used. 100 g. faeces, 100 g. soil and finally 50 g. bran mixed with 50 g. oats were each suspended in 1 l. water containing urea, phosphate, glucose and $FeSO_4$ in amounts similar to those added to the rumen contents (Fig. 1). With the faeces the apparent increase in protein was much greater than with the rumen contents, and the increase was rapid from the beginning of the incubation, whereas with the rumen contents there was an initial lag of 2–3 days. The explanation may be that the bacteria present in the faeces in such large numbers consist of types for which the stagnant faecal suspension is an ideal medium and that the faeces contain few, if any, of those types present in the rumen which die so readily when removed thence and which are very difficult to grow on the usual types of media. It is therefore not surprising that in these prolonged incubations there is an initial lag period with the rumen liquor but not with the faeces.

The soil suspensions were by no means sterile, for they brought about a marked conversion of urea to NH_3 and of NH_3 to nitrate (Fig. 1). No change in N.P.N. could, however,

be detected. The reason is probably that the number of bacteria found in soil suspensions of this kind is so small compared with the numbers present in the faeces and rumen contents that they could multiply very greatly and utilize N.P.N. without causing any measurable decrease in the N.P.N. of the medium. These three types of experiment suggest therefore that the chemical changes denoting increase in protein when faeces and rumen ingesta are incubated may be associated with the micro-organisms they each contain.

With bran and oats the results were more complex. Fermentation set in early and was accompanied by much frothing and a very foul smell. During the first 6 days fermentation resulted in a considerable decrease in the protein content (Fig. 1). After that, however, the protein increased again but never attained the original value. This increase at the later stages was possibly due to some strains of bacteria passing from a stationary phase to a phase of more active growth.

Short-period incubations. Samples from the long-period incubations described above were examined microbiologically by Baker [1942 *a*, *b*; 1943] who stated that even after 1 day's incubation great changes had taken place in the microflora and fauna, so that the 'microbiological picture' at the end of 1 day bore little resemblance to that in the initial sample. Experiments were therefore carried out to determine how long samples of rumen contents could be incubated without detectable microbiological change. Samples of rumen ingesta from these experiments were examined by Mr Baker who found no significant change during the first 2–4 hr. of incubation. In all subsequent work therefore incubation periods of 2–4 hr. were adopted, since it was essential, if the work were to have any practical bearing, that any protein synthesis detected should occur under conditions as similar as possible to those in the intact rumen (Fig. 2).

Approximately 1500 ml. rumen liquor were incubated with 0·65 g. urea, 4·5 g. K_2HPO_4, 0·1 g. $FeSO_4$ and 7·5 g. starch. The *p*H was controlled when necessary by the addition of a few drops of concentrated Na_2CO_3 solution. From Fig. 2 the following facts appear. (1) The total N remained unchanged. (2) Urea was rapidly converted to NH_3. (3) Relatively little N.P.N. was present except as urea or NH_3. (4) During the first 3 hr. when the microbiological conditions in the medium appeared to resemble closely those of the rumen, synthesis of protein appeared to occur to the extent of about 9 mg. N/100 g. rumen liquor. (5) There was a decrease in N.P.N. accompanied by a parallel decrease in ammonia N, which suggests that the synthesized protein was produced from the ammonia N and not from the N.P.N. present in other forms.

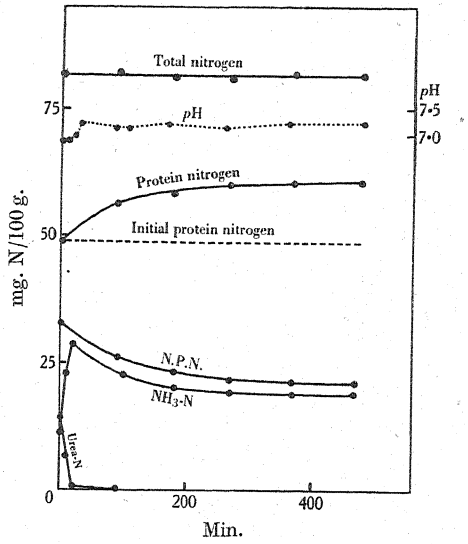

Fig. 2. The results obtained in a typical short period incubation of rumen liquor.

Table 1. *Comparison between sodium tungstate and trichloroacetic acid methods of estimating N.P.N.*

mg. *N*/100 g. rumen contents

Incubation no.	Initial N.P.N.			Final N.P.N.			Synthesis	
	Tungstate	Trichloroacetic acid	Difference	Tungstate	Trichloroacetic acid	Difference	Tungstate	Trichloroacetic acid
1	120·1	128·7	8·6	111·3	119·8	8·5	8·8	8·9
2	112·0	119·4	7·4	107·8	116·4	8·6	4·2	3·0
3	115·0	124·6	9·6	111·7	120·3	8·6	3·3	4·3
4	113·4	119·8	6·4	106·6	115·2	8·6	6·8	4·6

Table 2. *Protein synthesis estimated by the tungstate and trichloroacetic acid methods*

Total N = 143·5 mg./100 g. rumen liquor

Time min.	Tungstate method		Trichloroacetic acid	
	N.P.N. (initial)	Synthesis	N.P.N. (initial)	Synthesis
	(mg. N/100 g. rumen liquor)			
Initial	94·9	—	107·8	—
20	93·7	1·2	105·9	1·9
80	88·8	6·1	103·5	4·3
90	88·1	6·8	100·6	6·2
100	87·6	7·3	99·7	8·1
180	87·4	7·5	98·0	9·8
290	86·9	8·0	99·6	8·2

This amount of protein synthesis appears at first sight to be very small. If, however, the contents of the rumen be estimated conservatively at 75 kg., this value of 9 mg. N/100 g. rumen contents in 3 hr. is equivalent to the conversion of 54 g. N to 337 g. protein in 1 day. 54 g. N would be equivalent to some 25 % of the total protein requirements of a cow yielding 2–3 gallons of milk per day.

Control experiments were carried out with bran and oats, faeces and soil but no change in N.P.N. was ever found during the first 3 hr. of incubation.

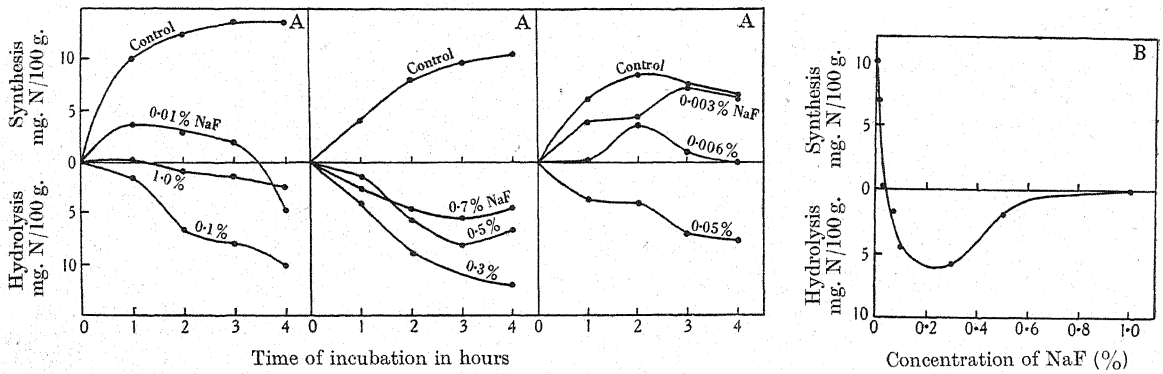

Fig. 3. A, the amount of synthesis or hydrolysis observed with different concentrations of NaF compared with controls incubated in the absence of NaF (first NaF experiment). B, the amount of synthesis or hydrolysis observed after incubation for 4 hr. with different concentrations of NaF (second NaF experiment).

The apparent synthesis observed with rumen contents seems therefore to be peculiar to the rumen ingesta.

Synthesis of some protein has always been observed in normal incubations of this type, both with tungstate and the CCl_3.COOH procedure outlined in a previous paper [Pearson & Smith, 1943*a*]. From Tables 1 and 2 it will be observed that CCl_3.COOH gave values for N.P.N. which were 6–13 mg. higher than those obtained by acid sodium tungstate, but nevertheless the amount of protein synthesis estimated by the two methods was of the same order.

When the occurrence of a decrease in N.P.N. in such incubations had been repeatedly confirmed, the question arose whether this was due to protein synthesis or to some other cause. If synthesis were the explanation, questions arose as to whether it was indeed due to micro-organisms, and whether its magnitude was affected by the presence of carbohydrates, proteins and amino-acids.

The microbiological nature of the synthesis

Proof that the changes in N partition observed on incubation were biological in origin was first obtained.

Influence of sodium fluoride. The first method of approach was to study the effect of incubating rumen liquor with certain toxic substances such as NaF. In a typical experiment 2·4 l. rumen liquor were divided into 4 equal parts. One part was used as a control while to the other 3 parts NaF was so added that one contained 0·01 %, another 0·1 % and a third 1 %. To all 4 parts, 100 ml. solution containing 1 g. urea and 3 g. starch were added. These mixtures were then incubated for 4 hr. with a slow stream of CO_2 passing through them. Samples were removed for analysis initially and at the end of each hour (Fig. 3, A). In two other similar experiments the concentrations of NaF were 0·003, 0·006, 0·05, 0·3, 0·5 and 0·7 %. The data in Fig. 3 show that protein synthesis was strongly inhibited by as little as 0·01 % NaF, and that with a concentration of 0·1 %, protein synthesis was replaced by hydrolysis. With 1·0 % hydrolysis was almost inhibited in the first instance and completely inhibited in the second.

When two 500 ml. portions of the same rumen liquor were incubated for 3 hr. with 1·8 g. starch and 0·3 g. urea, one containing 0·5 % NaCl, the other 0·5 % NaF, the results showed that 0·5 % NaCl had no effect on the synthesis occurring in the 3 hr., whereas with 0·5 % NaF (Fig. 3) hydrolysis predominated over synthesis. The action of the NaF was therefore a specific one associated with its recognized inhibitory action on enzymes and micro-organisms.

Table 3. *Protein synthesis in presence of NaCl*

	Control mg. N/100 g.		0·5 % NaCl mg. N/100 g.	
	Total N	N.P.N.	Total N	N.P.N.
Initially	103·5	33·0	103·7	33·4
After 3 hr.	103·3	27·3	103·4	27·4
Synthesis	—	5·7	—	6·0

It appears therefore that the change during incubation in the difference between total N and N.P.N. is the resultant of two different processes, one synthetic, the other hydrolytic, and that both are of microbiological origin. As the concentration of NaF increases, synthesis diminishes and becomes completely masked by hydrolysis. When, however, the concentration of NaF increases above 0·2 or 0·3 % hydrolysis also wanes; ultimately, at a concentration of 1–2 % NaF, the rumen liquor becomes 'fixed'.

Table 4. *The effect of quinone on protein synthesis*

Quinone %	Synthesis (+) or hydrolysis (−) mg. N/100 g.
None	+12·2
0·001	+ 8·5
0·005	+ 5·4
0·01	+ 5·1
0·05	− 6·4
0·01 + H_2S	+10·5
H_2S + water alone	+10·9

Table 5. *The effect of boric acid and borax on protein synthesis*

Conc. of boric acid or borax %	Synthesis mg. N/100 g. rumen liquor: Sample 1 Boric acid	Sample 1 Borax	Sample 2 Boric acid	Sample 3 Borax
Control	9·1	9·1	5·9	14·0
0·01	—	—	5·8	10·2
0·03	—	—	3·0	—
0·05	3·6	2·2	—	—
0·06	—	—	4·2	—
0·10	1·4	1·6	3·2	4·7
0·30	—	—	−0·2*	0·9
0·50	0·5	0·2	—	—
1·00	—	—	0·0	−3·0*
2·00	—	—	—	−4·4*

* Hydrolysis.

Influence of quinone. A similar experiment was made with quinone alone and with quinone in the presence of H_2S (Table 4). Synthesis was markedly diminished in the presence of even 0·005 % quinone, and at a concentration of 0·05 % hydrolysis predominated. In the presence of H_2S the quinone appeared to lose its toxic properties. This agrees with the behaviour of quinone in the presence of thiol compounds previously recorded for soya bean urease by Quastel [1933], and also observed with rumen-urease activity by Pearson & Smith [1943*b*].

Influence of boric acid. Two experiments similar to those with NaF and quinone were performed, one with boric acid and the other with borax. Table 5 shows that synthesis was again greatly diminished and that with the two highest concentrations of borax hydrolysis predominated.

Influence of temperature. All the above experiments suggested that the observed protein synthesis was the result of biological processes. If so, changes in temperature might also be expected to affect the magnitude of the synthesis. Different portions of the same sample of rumen liquor were therefore incubated with urea and starch for 1½ hr. at temperatures varying between 0 and 90°. Fig. 4 shows

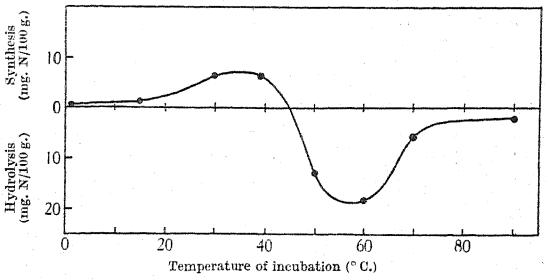

Fig. 4. The amount of synthesis or hydrolysis obtained by incubating rumen liquor for 1½ hours at different temperatures.

that at temperatures below 16° little change occurred, but that at 30 and 40° the usual amount of protein synthesis took place. When, however, the incubation temperature was 50° hydrolysis predominated. Hydrolysis attained a maximum at about 60° and was almost entirely inhibited at 90°. From 40 to 90° the curve is very similar indeed to that obtained with increasing concentrations of NaF (Fig. 3), except that hydrolysis in the absence of NaF attained a greater magnitude at 60° than was observed with any concentration of NaF at 39°. This difference may be due to the use of different samples of rumen contents in the two instances. However, the higher temperatures (up to 50 or 60°) may greatly inhibit synthesis but may actually increase the hydrolytic processes, whereas NaF tends to inhibit both, the more sensitive synthetic process disappearing first.

Influence of toluene. Pearson & Smith [1943*b*] have shown that toluene has no effect on the urease activity of the rumen liquor. A test was therefore carried out to see whether synthesis is affected by this substance. Two 500 ml. portions of the same sample of rumen liquor were incubated with starch and urea for 3 hr. To one sample were added 50 ml. toluene. The control gave a synthesis of 7·4 mg. N/100 g. rumen liquor, whereas with toluene hydrolysis occurred amounting to 13·7 mg. N/100 g. This again shows how readily synthesis can be diminished, allowing hydrolysis to predominate. The urease activity in this instance remained unaffected.

Effect of filtration. Rumen liquor was filtered through a Chamberland filter type 'F'. The clear filtrate was then incubated in the presence of 0·4 % starch and approximately 0·5% NH_4HCO_3. Since

Pearson & Smith [1943*b*] had already shown that a filtrate of this type possessed no urease activity, the N was added in the form of NH_4HCO_3 and not as urea. In the absence of urease activity noted in previous work it was not surprising that neither synthesis nor hydrolysis was detected in this experiment, which again suggests that the synthetic and hydrolytic processes consistently observed in these incubations are to be associated with the micro-organisms of the rumen.

The effect of fractionation by centrifuge. On allowing the rumen liquor to stand for even a very short time, the Protozoa and the larger bacteria tend to sediment. On centrifuging for 5 min. at 1000 r.p.m. this tendency is very marked indeed. The bottom layer consists of a sludge rich in Protozoa and large bacteria which stain blue with iodine [Baker, 1942 *a*, *b*; 1943]. An experiment was therefore carried out to see whether the amount of synthesis was greater or less in the top and bottom fractions of the rumen liquor than in the sample of rumen liquor as a whole. At the same time the effect of keeping the rumen liquor and its fractions at approximately 0° for 24 hr. before incubation, was studied. A 3 l. sample of rumen liquor was centrifuged in 250 ml. cups for 5 min. at 1000 r.p.m. The contents of two of the cups were then thoroughly remixed by shaking. One portion of the remixed ingesta was incubated with 1 g. starch and 0·5 g. urea as the control. The other was first kept in the refrigerator for 24 hr. and then treated in the same way. 50 ml. of the liquor at the top and 50 ml. of the sludge at the bottom were then removed from each of the ten remaining centrifuge cups. The top fractions were mixed together and half of the sample incubated immediately with starch and urea, the other half being placed in the refrigerator for 24 hr. before incubation. The bottom fractions were similarly treated. From the results in Table 6 two main facts emerge. First, that

Table 6. *Protein synthesis with two fractions of rumen liquor separated by centrifuging*

	Synthesis (+) or hydrolysis (−) mg. N/100 g.	
	Immediately	After 24 hr. at 0°
Rumen liquor	+11·1	+2·8
Top fraction	+14·7	+5·0
Bottom fraction	+ 7·2	−2·5
Arithmetical mean of top and bottom fractions	+10·9	+1·3

holding the rumen contents at 0° for 24 hr. greatly reduces the amount of synthesis not only in the total rumen liquor but also in the top and bottom fractions—a fact which again suggests that this synthesis is associated with certain of the micro-organisms present in the rumen which are readily inactivated. Secondly, that on dividing the rumen liquor into 2 fractions by centrifuging, the top portions show very much greater amounts of synthesis than the bottom portions, whether the various liquors are incubated immediately or after 24 hr. at 0°. The possible significance of this finding is discussed later.

From all these experiments it appears that when rumen liquor is incubated with starch and urea at 39° the N metabolism of the system involves both synthetic and hydrolytic processes which probably proceed simultaneously. These processes are microbiological in origin and the equilibrium between them is very readily changed, so that either synthesis or hydrolysis predominates according to the conditions during the incubation. This last point is of great importance in connexion with urea utilization, for if the processes in the rumen at any time are predominantly hydrolytic with NH_3 as the end-product, the utilization, if any, must be due to processes other than the conversion of these substances to protein in the paunch. Factors which might be expected to influence the magnitude of the synthesis and its predominance over hydrolysis were therefore investigated next.

Influence of p*H.* The *p*H of the rumen ingesta varies slightly from time to time but it is rarely below 6·3 or above 7·4. In many of the incubations carried out during this work *p*H determinations have been made, and the values have usually been between 6·3 and 7·4, even after incubation for 3–4 hr. at 39°. No correlation was found between the *p*H during incubation and the amount of protein synthesis, provided the *p*H lies roughly between the above limits. Thus three samples of the same rumen liquor were incubated for 5¼ hr. with 4 g. K_2HPO_4, 0·1 g. $FeSO_4$, 5 g. glucose, 5 g. starch and approximately 1 g. urea/l., CO_2 being passed through each flask throughout the incubation period. In the case of the 1st sample (M) the *p*H was measured at frequent intervals and adjusted by adding the correct amount of a saturated solution of Na_2CO_3. To the 2nd sample (N) the same amount of Na_2CO_3 was added at the same times as to M, without previously removing samples to measure the *p*H. To the 3rd sample (P) 10 g. powdered $CaCO_3$/l. were added initially. Fig. 5 shows that the *p*H in the first two samples was fairly close to the figure of 7·2 throughout, whereas in the 3rd sample the initial value of 7·2 decreased during the incubation to 6·3. Solid $CaCO_3$ is therefore of little value in maintaining the initial *p*H. The amount of synthesis in M could not be measured accurately since so many samples were removed to measure the *p*H before and after adjustment, but the results for N and P show that the amount of synthesis when the *p*H remained constant did not

differ significantly from the amount observed when the *p*H fell from 7·2 to 6·3.

In the previous papers of this series the great buffering power of the rumen liquor is discussed. In view of this it seemed unnecessary to study the effect on synthesis of hydrogen-ion concentrations outside the normal range of 7·4 to 6·3, particularly

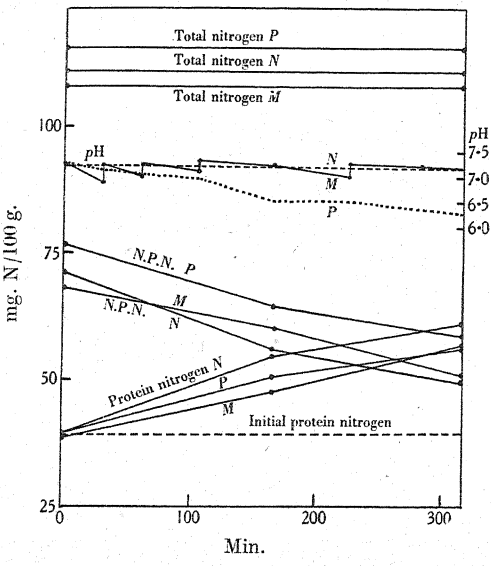

Fig. 5. The amount of synthesis obtained when the *p*H is kept constant at 7·2 compared with that obtained when the *p*H is allowed to decrease from 7·2 to 6·3.

as it was found that synthesis may be affected as much by the type of buffer employed to obtain the *p*H as by the *p*H itself. That synthesis can be markedly affected, however, by an abnormally high *p*H is shown in the experiments which follow, in which incubations were carried out in the presence of different gases.

Influence of CO_2-N_2. The composition of the gas in the rumen varies slightly from time to time, but typical analytical figures such as those found by Olson [1940] are: CO_2 62%, CH_4 15%, N_2 18% and O_2 1%. In view of the great preponderance of CO_2 and the low concentration of O_2 in these gases, experiments were carried out to find whether the nature of the gas passing through the medium had any influence on the amount of synthesis. One sample of rumen liquor was divided into 6 portions. Three of these were incubated for 3 hr. with 3 g. of starch and 800 mg. urea-N/l. while the other 3 were incubated with starch only. There were thus 3 pairs of samples, one in each pair containing added urea and the other containing no urea. A slow stream of CO_2 was passed through the 1st pair, N_2 through the 2nd and air through the 3rd. From the results in Table 7 the following points emerge. (1) Without added urea, protein synthesis in the presence of CO_2 was so little greater than that with N_2 and air that it is doubtful if it was significant. (2) When approximately 800 mg. urea-N/l. were present, protein synthesis in the presence of CO_2 was substantial, but with N_2 and air hydrolysis predominated. (3) Hydrolysis occurred with N_2 and air perhaps because with these two gases the *p*H increased during the incubations to 8·7, whereas with CO_2 it remained between 6·8 and 6·6. To see whether this predominance of hydrolysis was due to the nature of the gases or to the change in *p*H, a second experiment was carried out in which the amount of urea-N added to 3 of the 6 flasks was only 150 mg./l. In this instance the *p*H of the N_2 and air samples did not exceed the initial value of 6·9. From Exp. 2 (Table 7) the following conclusions may be drawn. (1) In the absence of added N.P.N. synthesis in air and in N_2 was not significantly less than that obtained with CO_2. (2) With an amount of urea insufficient to cause an appreciable change in *p*H when the urea was converted to NH_3, CO_2 and N_2 gave very similar amounts of synthesis. But with air there was significantly less. It is clear from these two experiments that the hydrolysis in air and in N_2 in Exp. 1 was due to the rise of *p*H and not primarily to the gases themselves. From many similar experiments the authors concluded

Table 7. *Effect of different gases on protein synthesis*

		Exp. 1		Exp. 2	
		*p*H	Synthesis (+) or hydrolysis (−) mg. N/100 g.	*p*H	Synthesis (+) mg. N/100 g.
Without added urea	CO_2	6·8–5·9	+ 3·9	6·9–5·9	+4·9
	N_2	6·8–6·3	+ 2·1	6·9–6·3	+4·6
	Air	6·8–6·6	+ 2·1	6·9–6·2	+4·7
80 mg. urea N/100 g. rumen liquor in Exp. 1 and 15 mg. in Exp. 2	CO_2	6·8–6·6	+14·0	6·9–6·1	+7·9
	N_2	6·8–8·7*	− 9·2	6·9–6·6	+8·2
	Air	6·8–8·7*	− 4·2	6·9–6·8	+5·4
No urea; 0·1 g. K_2HPO_4/100 g.	CO_2	—	—	7·1–6·1	+5·0

* At *p*H 8·7 NH_3 escapes from the medium during the incubation. In estimating the amount of hydrolysis the loss in total nitrogen is therefore allowed for.

that the greatest synthesis was always obtained when CO_2 was passed slowly through the medium, CO_2 having the advantage over N_2 that in the presence of reasonably small amounts of urea or NH_4HCO_3 it prevented an unfavourable increase in *p*H.

In Exp. 2 (Table 7) the *p*H of the sample incubated with CO_2 with no added urea decreased from 6·9 to 5·9. Hence protein synthesis might have been significantly greater than 4·9 mg. N/100 g. had the *p*H not diminished so far. A 7th incubation was therefore carried out simultaneously in which 0·1 g. K_2HPO_4/100 g. rumen contents was present. The *p*H in this instance diminished only to 6·1 but protein synthesis remained unchanged.

Influence of dietary factors on the utilization of non-protein nitrogen

NH_4HCO_3 and $(NH_4)_2SO_4$ as urea substitutes. Because urea is now indispensable to many industries it is less readily available as an animal feeding-stuff. NH_4HCO_3 and $(NH_4)_2SO_4$ are more plentiful and more readily prepared on a large scale. Incubations were therefore carried out to see whether with these substances protein synthesis equalled that obtained with urea. 3 g. starch were present per l. rumen liquor in every instance, and a slow stream of CO_2 was passed through all the flasks. Incubation lasted 2 hr. Two series of experiments were made. In one the amount of N.P.N. added in the different forms was 90–100 mg./100 g., in another it was about 15 mg./100 g. It can be seen from the results (Table 8), first, that the amount of synthesis with these particular samples of rumen liquor was increased by the addition of N.P.N. and secondly, with NH_4HCO_3 it was equal to that with urea and greater than that with $(NH_4)_2SO_4$. It is not clear why sulphate should be less efficient than bicarbonate in promoting protein synthesis. This difference was, however, only significant in the first instance when a relatively large amount of sulphate was added, but in practice the quantity of $(NH_4)_2SO_4$ fed to a lactating cow to replace a portion of the dietary protein would be equivalent, if given in two equal portions per day, to only about 25 mg. N/100 g. of rumen contents, even immediately after feeding. It is doubtful, therefore, if there would be any detectable difference in the efficiency of the utilization of these three different forms of N. On the basis of the present results, however, preference should be given to urea or NH_4HCO_3.

Influence of urea concentration. In order to see whether urea concentration had much effect on the amount of protein synthesis a series of incubations was carried out in which 1 large sample of rumen liquor was divided into 7 equal portions and different amounts of urea added to each. Incubation lasted 3 hr. in a slow current of CO_2; 3 g. starch were added per l. rumen liquor. Table 9 shows that under these conditions the maximum amount of synthesis is obtained when the added urea-N amounts to 75 or 100 mg./100 g. Below and above these concentrations the amount of synthesis was significantly less. Since urea is so rapidly converted to NH_3 in the rumen liquor, these results should be equally true for NH_4HCO_3.

Table 8. *Protein synthesis with urea, NH_4HCO_3 and $(NH_4)_2SO_4$**

	Exp. 1			Exp. 2		
Supplement	N.P.N. added (mg. N/100 g.)	Protein synthesized (mg. N/100 g.)	*p*H	N.P.N. added (mg. N/100 g.)	Protein synthesized (mg. N/100 g.)	*p*H
—	—†	4·0	6·7–5·7	—†	1·3	6·5–5·6
Urea	100	11·5	6·7–6·8	16	5·1	6·5–5·9
NH_4HCO_3	80	11·3	7·2–6·7	15	5·4	6·5–5·6
$(NH_4)_2SO_4$	85	6·3	6·5–5·8	15	4·5	6·5–5·9

* Analar. † N.P.N. of rumen contents with no addition was initially 11·4 in Exp. 1 and 6·9 in Exp. 2.

It early became clear that the utilization of N.P.N. by the ruminant may depend on the nature of the diet as a whole, and particularly on whether the entire ration is correctly balanced. If adding urea upsets the proper proportions of protein and potential protein to carbohydrate, the urea itself may not be efficiently utilized. This may explain some of the conflicting results cited in Krebs's [1937] review and which have been obtained from time to time during the past 40 years in experiments in which N.P.N. has been added to the diet of rumi-

Table 9. *Influence of concentration of urea on protein synthesis*

Urea added (mg. N/100g.)	Synthesis mg. N/100 g.	Final *p*H*
—	5·2	5·7
50	7·8	6·2
75	11·3	6·4
100	11·2	6·6
150	6·4	7·1
400	6·7	7·6
650	2·0	7·8

* The initial *p*H in this instance was 6·2 throughout.

nants. Presumably the greatest efficiency of urea as a partial protein substitute would be shown when the dietary protein equivalent was low compared with the starch equivalent. In such a diet starch itself would probably be present in relatively large amounts. The effect of different concentrations of starch and also of different carbohydrates on the amount of protein synthesis observed in the *in vitro* incubations was therefore studied. The results were of such interest that a further series of experiments was made in which the effect of different proteins and amino-acids was studied.

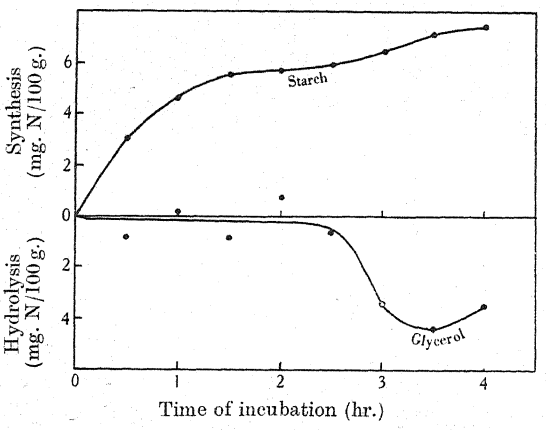

Fig. 6. The synthesis obtained by incubating rumen liquor in the presence of 0·3% starch compared with the hydrolysis obtained when starch is replaced by glycerol of the same concentration.

Influence of concentration and nature of carbohydrates. Preliminary tests showed that when 3 g. starch/l. were added to the rumen liquor the amount of synthesis was always greater than when rumen liquor was incubated alone. Moreover, when starch was replaced by glycerol synthesis was very restricted in amount and was sometimes replaced by hydrolysis. Typical results for an experiment in which two samples of the same rumen liquor were incubated, one with starch and one with glycerol (both 3·6 g./l.) are shown in Fig. 6. 2·4 g./l. urea were present and CO_2 was passed through the samples during incubation. Whereas with starch protein synthesis was equivalent to 7·4 mg. N/100 g., glycerol was responsible for hydrolysis amounting to 3·5 mg. N/100 g. Four separate portions of the same rumen liquor were therefore incubated at one time with different carbohydrates (Exp. 1, Table 10). One contained 3 g. starch/l., the 2nd 3 g./l. galactose, the 3rd sucrose and the 4th maltose. Similar comparisons of the influence of starch with that of other carbohydrates were also made. Thus the protein synthesis obtained in every instance could be related to that obtained with starch. Only the average amounts of synthesis in this first experiment are shown in Table 10, as the average values are so typical of all the results that there is no necessity to quote the individual values. But for Exps. 2–4 (Table 10) in which 7 incubations were carried out at one time, the individual results are quoted. In all these experiments no urea was included in the diet of the steer, and since in Exp. 1 (Table 10) none was added to the rumen liquor, the initial N.P.N. was very low, 4–10 mg. N/100 g. In Exps. 2–4, however, the N.P.N. was much higher, due to the addition of about 100 mg. urea-N/100 g. rumen liquor in each incubation. Presumably the greater amount of synthesis in the three latter experiments was due to this. The results, which confirm the fact that synthesis and hydrolysis are progressing simultaneously, but that synthesis predominates under certain conditions, lead to the following conclusions. (1) That the amount of synthesis depends to some extent on the starch

Table 10. *Protein synthesis in presence of carbohydrates*

	Exp. 1			Synthesis (mg. N/100 g.)			
Carbohydrate	Wt. added g./l.	Average synthesis (+) or hydrolysis (−) mg. N/100 g.	No. of determinations	Exp. 2	Exp. 3	Exp. 4	Final *p*H*
Starch	10	+3·7	2	—	—	13·7	7·0
,,	7	+3·5	2	—	—	13·3	6·9
,,	3	+4·0	18	13·7	14·1	10·9	6·9
,,	1	+1·9	2	—	—	5·7	6·9
,,	0·5	−1·9	2	—	—	4·5	6·6
No addition	—	−3·3	6	—	0·2	−2·0	6·4
Galactose	3	+3·5	5	10·5	10·5	—	—
Sucrose	3	+1·3	5	9·6	—	—	—
Maltose	3	+3·7	4	9·4	9·3	—	—
Lactose	3	+2·7	3	3·2	—	—	—
Glucose	3	+1·6	4	3·5	3·4	—	—
Dextrin	3	+0·5	5	—	0·1	—	—
Glycerol	3	−0·4	6	3·7	—	—	—
Lactic acid	3	−2·0	3	—	1·6	—	—

* The initial *p*H was 7·0 in all incubations.

concentration, e.g. in the absence of starch an hydrolysis of 2·0 mg. N/100 g. was observed, but in its presence synthesis predominated. In Exp. 4 protein synthesis attained a maximum at a starch concentration of 7 or 10 g./l. On the average, however, the synthesis with 7 and 10 g. starch/l. rarely exceeded that obtained with 3 g./l. Below 3 g./l. synthesis decreased significantly. (2) That the amount of synthesis depends on the nature of the carbohydrate added, being always greatest with starch, galactose and maltose; dextrin, glucose, glycerol and lactic acid being all relatively poor stimulants. (3) That with all these substances protein synthesis was much greater with, than without, urea.

Influence of amino-acids and proteins. In this section only preliminary experiments have been completed. The results are therefore presented tentatively. Rumen liquor was incubated with various amino-acids and proteins. In all instances 3 g. starch/l. were present and CO_2 was passed through the medium. The period of incubation was 3 hr. The data for Exp. 1 (Table 11) show that arginine was particularly active in promoting synthesis, whereas tyrosine had little, if any, effect.

Table 11. *Protein synthesis or hydrolysis in presence of amino-acids*

	Synthesis mg. N/100 g. rumen liquor		
No. of exp. ...	1	2	3
No addition	4·2	3·8	2·3
Glycine	6·0	—	—
Alanine	—	4·3	—
Valine	—	3·5	5·6
Leucine	8·9	—	—
Lysine	—	1·0	4·6
Histidine	7·9	1·8	—
Arginine	13·2	—	—
Tyrosine	5·5	—	—

Table 12. *Protein synthesis or hydrolysis in presence of leucine and asparagine*

Leucine		Asparagine	
Added %	Synthesis (+) or hydrolysis (−) mg. N/100 g.	Added %	Synthesis (+) or hydrolysis (−) mg. N/100 g.
None	+2·7	None	+4·8
0·47	−0·7	0·62	+1·1
1·13	−2·0	1·25	−4·5
2·26	−0·7	2·5	−1·2
0·47 + 0·5 urea	+6·6		
0·5 urea alone	+3·0		

How far this is due to arginase activity in the rumen will be investigated in future work. Histidine and leucine were effective but much less so than arginine. These results, however, were not confirmed in the two subsequent experiments. Thus histidine appeared to cause a decrease in protein synthesis (Table 11) and leucine actually brought about hydrolysis unless urea was present with the amino-acid (Table 12). The explanation is not at present clear. Perhaps some amino-acids only promote synthesis in certain samples of rumen liquor or when certain other non-protein nitrogenous constituents are present. Further work may clear this up. Table 12 shows that asparagine causes hydrolysis rather than synthesis.

Table 13. *Protein synthesis or hydrolysis in presence of protein or substances rich in protein*

Protein added	%	Synthesis (+) or hydrolysis (−) mg. N/100 g. rumen liquor	
		Sample 1	Sample 2
None	—	+5·0	+ 4·0
Blood meal	0·1	+5·2	—
Egg white	—*	+1·0	—
Casein	0·1	−6·4	—
Gelatine	0·2	—	−12·5
Gelatine	0·5	—	−27·2
Gelatine	1·0	—	−47·0

* Equivalent to about 0·1 % albumen.

In two other experiments with casein in which different samples of rumen contents were used, hydrolysis was 3·5 and 31·3 mg. N/100 g. compared with synthesis of 7·7 and 10·4 mg. N/100 g. respectively in the controls.

The results of preliminary experiments with four proteins are recorded in Table 13. With blood meal the amount of synthesis was the same as that in the absence of added protein. Fresh uncoagulated egg white gave a much reduced synthesis, whereas with soluble casein, prepared freshly from milk, hydrolysis predominated. Hydrolysis also prevailed when gelatine was present and increased with increasing amounts of this protein.

DISCUSSION

The object of this work has been to find whether there is any evidence for the conversion of N.P.N. to protein in the rumen ingesta of the ox. It has been shown that when the rumen ingesta are incubated *in vitro* the total N.P.N. usually diminishes during the first 3 hr. and that the decrease is caused entirely by a fall in the ammonia-N fraction of the N.P.N. It has not yet been proved that this decrease in ammonia-N actually denotes a synthesis of protein, but a decrease in N.P.N. with no change in the total N is usually regarded as evidence of protein synthesis. It has been shown that this supposed synthesis is characteristic of rumen contents but not of incubations with faeces, soil or feeding-stuffs,

and that it occurs *in vitro* during the first few hours of incubation when, as shown by Baker [1942 *a*, *b*; 1943], the microbiology of the medium closely resembles that of fresh rumen contents.

That this synthesis is microbiological in origin has been shown by incubations with various toxic substances such as NaF, quinone and boric acid in minute amounts which caused a decrease in synthesis which changed to hydrolysis as the concentration of the inhibitor increased; with the highest concentrations both synthesis and hydrolysis were inhibited. This indicates that in the rumen synthesis and hydrolysis of protein proceed simultaneously, and that either one or the other may predominate according to the conditions. Abundance of starch, for example, favours synthesis, whereas its equivalent of glucose, glycerol or lactic acid does not. Some substances, e.g. lactic acid and gelatine, may even promote conditions in which hydrolysis predominates. In such instances, however, hydrolysis is not accompanied by destruction of the urease activity of the rumen ingesta. This occurs only when hydrolysis predominates owing to the presence of small concentrations of toxic substances such as NaF, quinone or boric acid.

While the present work was in the early stages Wegner *et al.* [1940] demonstrated the conversion of inorganic N to protein by inoculating appropriate media with micro-organisms from the cow's rumen. But we have studied this problem not by the inoculation of media in which conditions may differ markedly from those in the rumen but by incubating the rumen liquor itself under conditions as similar as possible to those in the rumen. This was done originally because many of the micro-organisms most characteristic of the rumen do not survive in other media, and it seemed possible that these less viable organisms might be concerned in the utilization of N.P.N. Thus it was observed that on incubating rumen ingesta synthesis of protein appears to be accompanied by protein breakdown. Hence, it is clear that the problem of N.P.N. utilization by ruminants can ultimately be solved only when the different factors influencing these two processes have been studied. The centrifuging experiments (p. 158) strongly suggest that protein decomposition is associated particularly with the heavier micro-organisms which sediment rapidly, i.e. synthesis greatly predominates in the top layers of the centrifuged rumen liquor.

That either protein synthesis or protein breakdown may predominate, according to the conditions in the rumen ingesta, gives rise to at least two important considerations. (1) That N.P.N. may be of value to the animal only if it is fed in a correctly balanced diet in which the starch equivalent is sufficiently high to balance almost all the N present. (2) That for ruminants the biological value of a protein may depend not only on its amino-acid content but also on whether its presence in the paunch promotes protein synthesis or hydrolysis. Thus with blood meal, which is very insoluble in the rumen liquor and can be seen apparently intact in the rumen ingesta some hours after feeding, synthesis occurred in the *in vitro* incubations, but with other water-soluble proteins less protein synthesis, and even marked production of NH_3, took place. Possibly therefore blood meal, due to its insoluble nature, passes through the rumen practically unaffected and is digested in the small intestine as with non-ruminant animals, whereas other more soluble forms of protein may be broken down slightly but significantly to NH_3 as they pass through the rumen. To what extent a protein is broken down to NH_3 may partly depend on the nature and amount of the non-protein nitrogenous substances present at the time. Thus in the presence of leucine only (Table 11) slight hydrolysis to NH_3 took place, but when urea was also present synthesis predominated. Note that these suggestions are based on only one experiment and that they therefore require confirmation. Nevertheless such indications did appear from time to time. The value of N.P.N. to the ruminant may therefore be, that though it can be utilized for protein synthesis, under certain conditions it also diminishes protein breakdown.

SUMMARY

1. Many experiments have been carried out in which liquid rumen contents have been incubated under different conditions and with different substances.

2. During a 2–4 hr. incubation, when the microbiology appeared to be practically unaltered, the N.P.N. of the medium diminished while the total N remained unchanged, which suggests that synthesis of protein took place.

3. The amount of synthesis occurring in 2 hr. in the presence of 0·3 g. starch/100 g. rumen liquor was usually equivalent to about 8 mg. N/100 g. rumen liquor. If this rate were maintained for 24 hr. in the inact rumen there should be protein synthesis equivalent to 72 g. N or 450 g. protein.

4. That the synthesis is microbiological in nature has been shown by experiments with NaF, quinone and boric acid, and by incubations at different temperatures.

5. Relative amounts of synthesis in the presence of various carbohydrates have been studied. Starch appears to have the most stimulating effect.

6. Similar but preliminary experiments have been carried out with proteins and amino-acids.

7. Protein synthesis occurring during the incubation of rumen liquor is accompanied by protein breakdown. Either process may predominate ac-

cording to the general conditions or to the substances present. The possible bearing of this on the utilization of N.P.N. and on the biological value of proteins for ruminants is briefly discussed.

The authors wish to thank those who have helped them in these investigations; Dr M. P. Applebey of Imperial Chemical Industries Limited, Prof. A. C. Chibnall, F.R.S., and Dr N. C. Wright for their interest and encouragement; Mr Frank Baker for many invaluable discussions, particularly on the microbiological aspects; Dr P. S. Watts, M.R.C.V.S., for making the rumen fistula in the steer and for maintaining the animal in excellent condition for almost 4 years; and various members of the Institute staff; also the directors of Imperial Chemical Industries Limited for allowing a member of their staff (R. M. P.) to assist in this work at the Hannah Institute, and for a grant towards expenses.

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The Committee have resolved that the following extract from the programme of the 224th meeting of the Society should be placed on permanent record in the Journal

THE RESIGNATION OF PROFESSOR HARINGTON FROM THE POST OF SENIOR EDITOR

At the meeting of the Society at Oxford on 26 September 1942, the Chairman of the Meeting, Prof. R. A. Peters, spoke of Prof. Harington's impending resignation from the post of Senior Editor of the *Biochemical Journal* and reviewed the excellent service that Prof. Harington had given to the Society over a period of 15 years.

The Chairman then called upon Prof. F. G. Young, who moved the following formal resolution of thanks to Prof. Harington:

'The Members of the Biochemical Society have learned with regret of the impending resignation of Prof. C. R. Harington, F.R.S., from the post of Senior Editor of the *Biochemical Journal* on his appointment as Director of the National Institute for Medical Research (Medical Research Council). They recall with gratitude that Prof. Harington has served continuously on the Committee of the Society for the past 15 years, first as an Ordinary Member of the Committee (1927–9), then as Joint Honorary Secretary (1929–30), and since 1930 as an Editor of the *Biochemical Journal*. From January 1938, until the present time, Prof. Harington has filled the onerous position of Senior Editor, with chief responsibility for the production and editing of the *Journal*. The Members of the Society appreciate that such continuous and responsible service on their behalf has absorbed a substantial proportion of Prof. Harington's time and energy during the past many years, and they wish to express their sincere gratitude and thanks for this unselfish devotion to the interests of the Society over so long a period.

'Although the Members of the Biochemical Society greatly regret the necessity of Prof. Harington's resignation from the Editorship of the *Journal*, they nevertheless wish to express their sincere congratulations on the appointment which has deprived the Society and the *Journal*, for a time at least, of his more active services. They are sensible that his appointment denotes not only the eminence of Prof. Harington in his many fields of interest, but also the ever-growing importance of the subject of Biochemistry.

'The Members of the Biochemical Society wish therefore to record their deep sense of gratitude for Prof. Harington's sustained devotion to the interests of the Society during the past years, to express their heartfelt congratulations on his appointment to a position of eminence in the field of Medical Research, and to convey their sincere good wishes for the years to come in the position of great responsibility which he now assumes.'

The resolution was adopted by the Meeting unanimously and with acclamation.

Obituary Notice

WILLIAM JOHN YOUNG (1878–1942)

Prof. W. J. Young, an original member of the Biochemical Society, died in Melbourne on 14 May 1942 after an operation for perforated gastric ulcer.

William John Young was born in Manchester on 26 January 1878 and was educated at the Hulme Grammar School and at Owens College where he specialized in chemistry and was awarded the M.Sc. degree of the Victoria University.

In 1901 he was appointed as assistant to Dr Arthur Harden at the Jenner Institute and thus began the long and fruitful association with Harden which was to add so much to our knowledge of the biochemistry of carbohydrates.

At that time Buchner's recent discovery of cell-free fermentation by yeast juice had focused attention on the behaviour of this organism, and Harden and Rowlands were engaged in studying its autofermentation. Young joined them and continued to work with Harden on the carbohydrates of yeast until he left the Lister Institute for Australia in 1912.

The six papers published in the *Transactions of the Royal Society* between 1906 and 1911 contain the story of the fundamental contribution of these authors to our knowledge of carbohydrate metabolism. Their outstanding discovery was the part played by phosphorylation in carbohydrate breakdown and the necessity for the intervention of both phosphate and co-enzyme in the process. It was Young who in 1907 isolated the barium salt of a

hexose-diphosphoric acid, and to Harden and Young we owe the conception of the co-ferment as a substance comprising both the phosphate radical and an organic residue of unknown structure capable of passing on the phosphate radical to the sugar molecule.

In temperament the two colleagues differed greatly, Young's enthusiastic nature contrasting with the reserve and caution which were characteristic of Harden. Young was an active partner in the work and certainly played no inconsiderable part in its development.

In 1912, a new Institute of Tropical Medicine had been established at Townsville, Australia, and a selection Committee was appointed in London under the Chairmanship of Dr C. J. M. Martin to appoint certain members of the Staff of the new Institute. Young was chosen as the Biochemist, and in 1912 Dr and Mrs Young with their daughter Sylvia left London to make a new home in Australia. Whilst at Townsville, Young took up the question of the alteration in metabolism produced by residence in the tropics, and some new observations were recorded in a series of papers published between 1915 and 1920, chiefly in the *Annals of Tropical Medicine and Parasitology*. In 1919, in collaboration with Dr Breuil, pathologist at the Institute, an interesting paper on Tropical Australia and its Settlement was written and appeared in the *Medical Journal of Australia*.

Chiefly owing to the exertions of Prof. W. A. Osborne, who had just left Townsville to take up the Chair of Physiology at Melbourne University, a Lectureship in Biochemistry was established at that University and offered to Young: somewhat later a Chair in Biochemistry was founded to which Young succeeded.

In Melbourne, Young's time was chiefly devoted to teaching; he was responsible for the instruction in this subject of students in the faculties of agriculture, dentistry, medicine, science and veterinary science; he built up a fine department, and in it were also carried out various investigations for the Government, two of especial interest dealing with the storage of fruit and the ripening of bananas.

Young was a man interested in many aspects of life. He was widely read in English literature, with a good knowledge of the drama of the Elizabethan age; he was also an expert but entirely self-taught cabinet maker. Whilst at the Lister Institute, he took up golf with his usual zeal and was to be found on summer evenings assiduously practising at Wimbledon Park. His colleagues at the Lister Institute remember him as an excellent friend, always good-tempered, interested in everything, and generally ready to argue on either side of the topic under discussion.

Within a period of two years the Biochemical Society has to mourn the loss of Harden, Robison and Young, the three men who did so much to unravel the mysteries of carbohydrate chemistry and to throw open this fruitful field to later investigations. I. S.-M.

The Vitamin A Content and Toxicity of Bear and Seal Liver

By K. RODAHL and T. MOORE, *From the Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council*

(*Received 23 October 1942*)

It has long been known among Eskimos and arctic travellers that the ingestion of polar-bear liver by men and dogs causes severe illness. It has also been reported that the liver of a certain seal (*Phoca barbata*) is poisonous, although opinion on this point is less unanimous.

Richardson [1861] recounts that when members of an expedition led by Barentzoon to Novaya Zembla in 1596 ate bear liver they all became ill. In three cases the illness was severe, with loss of skin from head to foot. Another early account of the phenomenon was given by Kane [1856], who experimented with bear liver and found the incidence of the poisoning to be inconsistent. On two occasions the whole company on a journey became sick after eating bear liver, but in other instances there were no ill effects. According to Koettlitz [1897], several members of an English expedition to Franz Josef Land, 1894–7, ate polar-bear liver and all suffered in consequence. Lindhard [1913] has also reported poisoning among members of another expedition. A bear was shot which, although thin, appeared to be healthy, and on the following day a stew was prepared from the liver, heart and kidneys. Although the heart and kidneys of bears had often been eaten without ill effects, the 19 men who partook of the stew all became sick. The first signs of distress occurred in two victims 2–4 hr. after the meal, and most of the others became ill during the night. The symptoms described

were drowsiness, sluggishness, irritability or irresistible desire to sleep, and severe headache and vomiting. During the second 24 hr. the skin of 10 out of 19 of the patients began to peel around the mouth, beginning in spots and gradually spreading over larger areas. In some cases the peeling was confined to the face, but in several it was general. Lindhard also described three other cases in which the skin peeled from head to foot after eating bear liver. The Norwegian explorer Nansen [1924] has mentioned that on two occasions he ate small amounts of bear liver without ill effects. It seems probable therefore that the poisonous effects only occur when large quantities are consumed. The most recent cases of poisoning have been described by Doutt [1940].

During a recent expedition made by one of us to north-east Greenland, 1939–40, specimens of polar-bear liver were collected with a view to identifying the toxic substance. On chemical and biological examination these specimens were found to be very rich in vitamin A, as also was a specimen of liver from *P. barbata*. It seems probable that this high concentration of vitamin A is the cause of toxicity, and that the ingestion of more than small amounts of liver leads to hypervitaminosis A.

METHODS

Vitamin A estimations. Specimens were brought from Greenland preserved in brine. Small portions were digested with alkali, and vitamin A was extracted according to the technique described by Davies [1933]. Vitamin A was then estimated by the $SbCl_3$ method, using a factor of 0·6 for the conversion of blue units into international units [Moore, 1937]. The first bear liver to be examined was taken from a 2-year-old female and contained 18,000 i.u./g. of wet material. A second specimen was taken from a 4-year-old male, and also contained 18,000 i.u./g. From a third specimen a value of 13,000 i.u./g. was obtained. A single specimen of seal liver contained 13,000 i.u./g. In biological tests groups of rats were given the liver oils in doses calculated to be equivalent to either 2·6 or 10·3 i.u. daily; other animals were given the international standard carotene at the same level. The weight increases observed were as follows:

	Calculated daily dose i.u.	Mean wt. increase in 28 days (g.)	Approx. i.u./g. liver by biol. test
Seal-liver oil (*Phoca barbata*)	2·6	25	14,000
	10·3	48	12,000
Bear-liver oil (no. 1) (*Ursus maritimus*)	2·6	29	26,000
	10·3	56	24,000
Carotene	2·6	23	—
	10·3	47	—

Since only a few rats were used (usually 3 males per group) these results do not allow accurate independent estimates to be made of the vitamin A contents of the livers. It is obvious, however, that they agree with the high contents found by the $SbCl_3$ method.

Tests for toxicity. According to Jackson [1899], V. Harley, of University College, London, examined bear liver in order to find the reason of its toxicity. Intraperitoneal and subcutaneous injections of alcoholic, ethereal and aqueous extracts had no toxic action on dogs and guinea-pigs, and a dog given an aqueous extract by mouth was also unaffected. Two mice died 3 days after subcutaneous inoculation with an ethereal extract, but the result was possibly accidental. Mice were unaffected by injections of alcoholic and aqueous extracts.

The very high content of vitamin A in the liver suggested that it might give rise to hypervitaminosis A if eaten in more than small amounts. This condition was first described by Takahashi, Nakamiya, Kawakimi & Kitasato [1925], and has since been investigated by many workers. Although it is not quite certain that vitamin A itself is poisonous, the toxicity is at least closely associated with the vitamin in its concentrates. In our laboratory the lesions produced have varied remarkably according to the size of the rat and the magnitude and duration of the overdosage. Skin lesions, ranging from a slight roughening of the hair to seborrhoea and alopecia, are common at all ages. When the vitamin is given in the form of drops of concentrate into the mouth, peeling of the skin at the corners is frequently observed. There may be enteritis, emaciation and pneumonia. More specific lesions, however, are softening and fracturing of the bones, seen most frequently in growing rats, and profuse and sudden internal haemorrhage, often seen in adult animals. In one series of experiments a dose of 30,000 i.u. vitamin A daily in the form of halibut-liver oil was found to be definitely excessive for rats of about 200 g. body weight, invariably causing roughening of the skin, and occasionally death through haemorrhage.

The liver tested for toxicity in the present experiments was from the 2-year-old female bear. Although a value of 18,000 i.u. of vitamin A/g. had originally been obtained the portions now tested contained only 10,000 i.u./g. Carefully planned tests were difficult because of the reluctance of the rats to eat the liver. We have observed the same disinclination in rats when given the livers of other rats which had been allowed to accumulate very high reserves of vitamin A. One rat ate a total of 33·1 g. of the bear liver during a period of 22 days, an amount containing an average of 15,000 i.u. of vitamin A/day. It became anaemic and the hind legs were paralysed. When moribund it was killed.

At autopsy, the profuse internal haemorrhage typical of hypervitaminosis A was found, particularly under the skin, but also in the pericardium. Another rat ate 5·3 g. of liver during a period of 9 days. It then accidentally cut a paw on the side of the cage and bled to death. On superficial examination the wound appeared too slight to have caused death in a normal animal. Another rat ate 24 g. of liver in 22 days without any ill effects. Two other rats which received smaller doses were also unaffected.

Attempts to fractionate the liver into toxic and non-toxic constituents were unsuccessful. An aqueous extract of the liver, and also the residue obtained after the removal of most of the vitamin A from the liver by extraction with alcohol, were readily consumed without ill effects, in amounts corresponding to 1–2 g. of fresh liver daily. Two rats given the residue after aqueous extraction of the liver, however, also sustained no injury, although almost all the vitamin A was contained in this fraction. It is probable that the absence of ill effects was due to the refusal of the animals to accept the liver in amounts sufficient to poison them.

DISCUSSION

It is questionable what factor should be taken to convert doses used for rats to the corresponding doses for man, but the ratio of 75 taken from the relative food intakes of the 100 g. rat and 70 kg. man would seem reasonable. If we take 100,000 i.u. of vitamin A as sufficient to cause immediate illness in the rat, then about 7,500,000 units should cause illness in man. This amount would be present in 375 g. of bear liver containing 20,000 i.u. of vitamin A/g., not an excessive portion to be eaten at a single meal.

Some years ago Mr J. F. Ward of the Crookes Laboratories mentioned to one of us a case of presumed poisoning through excessive halibut-liver oil consumption, which may be of interest in the present connexion. One of the men engaged in the manufacture of the oil took, without instruction, 4–5 oz. daily. After 5 days he became severely ill, the main symptom being giddiness. The ingestion of oil was then discontinued, and he rapidly recovered, returning to normal in 8–10 days. The daily dose must have been about 6,000,000 i.u.

In our tests with rats one animal succumbed with lesions specific for hypervitaminosis A. Other rats which ate almost as much of the liver showed no obvious sign of injury. If bear liver is toxic to the rat for any reason other than its high content of vitamin A, therefore, the amounts which must be eaten to cause poisoning must be so large as to render the animal liable to concurrent hypervitaminosis A. As far as can be concluded from experiments with rats, which may of course differ widely from man in their toleration of toxic substances, there seems no reason to look beyond vitamin A for the cause of toxicity in man although there is good evidence for the presence of other toxic substances in the tissues of arctic animals which would be unlikely to be rich in vitamin A. There would seem to be no objection to the use of preparations made from bear-liver oil as sources of vitamin A in therapeutic doses.

SUMMARY

1. Specimens of the livers of the polar bear and the seal, *Phoca barbata*, were found to be very rich in vitamin A. Three specimens of bear liver contained 18,000, 18,000 and 13,000 i.u./g. respectively of wet material. A specimen of the seal liver contained 13,000 i.u./g.

2. The ingestion of excessive amounts of bear liver by rats led in one instance to fatal hypervitaminosis A. Other rats which ate slightly smaller amounts of liver showed no obvious sign of injury. The well-known poisonous action of bear liver in man is probably due to its high content of vitamin A.

Our thanks are due to Dr Vernon Booth and Mr Alan Davies for help in parts of the work, to Dr L. J. Harris for his valuable criticism, and to the Scott Polar Research Institute for library facilities. One of us (K. R.) is indebted to the Nansenfondet, Oslo, Freiafondet, Oslo, and Varekrigs Forskningsfond, Oslo.

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Studies on Diffusing Factors

2. METHODS OF ASSAY OF HYALURONIDASE AND THEIR CORRELATION WITH SKIN DIFFUSING ACTIVITY

By D. McCLEAN, *From the Lister Institute of Preventive Medicine, Elstree, Herts*

(*Received 13 November 1942*)

Chain & Duthie [1940], who first described the association between diffusion in the tissues and the mucolytic activity of testicular extracts, reported good agreement in quantitative comparisons of the diffusing and viscosity-reducing activities of enzymes from many sources; they, as well as other workers, isolated a substance closely resembling hyaluronic acid from rabbit skin, which substance was acted on by these enzymes. On the other hand, Madinaveitia, Todd, Bacharach & Chance [1940] failed to obtain satisfactory correlation between diffusing and viscosity-reducing activities and suggested that it was not justifiable to assay diffusing factors in terms of their viscosity-reducing power. Hobby, Dawson, Meyer & Chaffee [1941] and Meyer, Chaffee, Hobby & Dawson [1941] studied the relationship between diffusing factors and hyaluronidase, and the character of these enzymes which were of bacterial and animal origin. The evidence these authors present indicates, that whereas hyaluronidase plays a part in the diffusion in tissues, the phenomenon is a complex one and cannot be explained on the basis of a simple chemical reaction. McClean & Hale [1941] pointed out that the environmental conditions of the test in the skin and those in the viscosimeter differ profoundly, and that such variable factors as salt concentration and *p*H markedly affect the apparent viscosimetric titre of these enzymes. They concluded that although at present it is certainly unjustifiable to assay skin-diffusing activity in terms of viscosity-reducing activity, it does not follow that the two activities are due to separate agents. Moreover, they reported that the inclusion of purified hyaluronic acid in the culture medium of *Cl. welchii* caused a marked increase, not only in the viscosimetric titre of the culture filtrate, but also in its diffusing activity. The same result was obtained with streptococcal cultures [McClean, 1941]. In view of the recognized specificity of such 'adaptive enzyme' effects, the fact that the presence of hyaluronic acid in the culture medium increases the production of diffusing factors is evidence for the view that this phenomenon is due to the activity of hyaluronidase.

Hyaluronic acid, when mixed with serum protein, gives a typical mucin clot in the presence of acetic acid [Seastone, 1939]; after treatment of the mucin with mucinase, however, this reaction no longer takes place. This phenomenon was used by Robertson, Ropes & Bauer [1940] to estimate the activity of the *Cl. welchii* mucinase, by observing the rate at which this primary degradation took place. It appeared that a comparative study of the activity of enzyme preparations from various sources with regard to three factors—inhibition of the precipitability of a hyaluronic acid-protein complex; reduction in viscosity of hyaluronic acid; and diffusing power in the skin—would be useful in elucidating the relationship between the successive stages in the degradation of the substrate. This paper includes a study of the mucin-clot prevention (M.C.P.) test, and the correlation between three methods of assay based on the above factors.

METHODS

Preparation of substrate. In the earlier experiments hyaluronic acid was obtained from umbilical cords by the method described by McClean & Hale [1941], which depends upon precipitation of an aqueous extract with 3 vol. of ice-cold ethanol containing a trace of Na acetate. The final product contains about 7% total N. It was found, however, that if the alcohol is saturated with K acetate and brought to *p*H 9–10 with KOH, the hyaluronic acid can be precipitated by the addition of only 1·25 vol. This precipitate, which forms a characteristic stringy clot, is broken up and thoroughly washed with alcohol to remove the residual K acetate and KOH. It is then washed with ether and dried over P_2O_5 *in vacuo*. This product contains considerably less protein than that derived by the original method and contains 4·8% total N, 2% P and 12% glucosamine. Its aqueous solution does not form a clot on the addition of acetic acid.

A 1% solution of twice-crystallized serum albumen in physiological saline was used for the preparation of the mucin-protein complex, since serum may contain variable amounts of an inhibitor or even traces of hyaluronidase [McClean, 1942]. The albumen was kindly supplied by Dr R. A. Kekwick and prepared by his method [Kekwick, 1938].

The substrate-protein mixture for use in the test is prepared as follows: 1 vol. hyaluronic acid solution is mixed with 1 vol. albumen solution; 2 vol. of distilled water are then added. The required strength of the hyaluronic acid solution for each batch of hyaluronic acid is determined by titrating representative enzymes with substrate-protein mixtures, prepared with both the original and the new substrate. The concentration of hyaluronic acid, which

gives the same end-point as that obtained with the original preparations, is used for all subsequent tests. It is found that the required concentration varies from 0·15 to 0·25% in distilled water. Solution of the hyaluronic acid is facilitated if the bottle is placed at 37° for 2–3 hr. and shaken occasionally. Stock solutions of hyaluronic acid and of albumen are kept in the refrigerator under toluene. The viscosity of hyaluronic acid solutions falls slowly on storage; solutions in which this fall has exceeded 5% of the original viscosity should not be used for the test, since it is known that the partially depolymerized substrate inhibits the activity of the enzyme.

Technique of the test. The test is performed at 100, 50 or 20% differences in dilution of the enzyme, but it is usual to do a preliminary test at a tenfold dilution to ascertain the approximate end-point of activity. The appropriate dilutions of the enzyme sample to be tested are made in a final vol. of 0·5 ml. distilled water. In order to obtain reproducible results if a final test at 20% differences is made, it is necessary to make these dilutions in bulk, say 10 ml., and to transfer 0·5 ml. amounts of the dilutions to the tubes for test. 1 ml. of the substrate protein mixture is added to each tube, including the control, starting from that tube containing the highest dilution of enzyme. The test mixtures are incubated for 20 min. at 37°. It is important that the period of incubation should be accurately controlled. Immediately after incubation the tubes are cooled in ice-cold water, to arrest enzymic activity and to assist clot production on the subsequent addition of acetic acid. After 5 min., 0·2 ml. 2*N* acetic acid is added to each tube. The tubes are held up to the light and gently shaken. The control, and those tubes containing little enzyme, show the development of a characteristic stringy clot which contracts on standing. One or two tubes with concentrations near the end-point may show a few threads, or some precipitate. Those tubes in which the enzyme has attacked the substrate show no clot or precipitate, and those in which the enzyme is present in considerable excess are clear. The highest dilution of the enzyme showing no clot or threads is taken as the end-point of activity.

Influence of concentration of substrate on apparent titre of enzyme

A preliminary test with an unpurified culture filtrate of *Cl. welchii* indicated that the apparent potency of the enzyme varies inversely with the concentration of the substrate mixture. This variation depends upon the concentration of the polysaccharide and, within wide limits, is independent of the concentration of protein. Observations were then repeated with purified enzyme derived from testis and from *Cl. welchii*. Substrate mixtures were prepared containing (1) double the usual concentration of polysaccharide and albumen, (2) the usual concentration, and (3) half the usual concentration. The salt content of the mixture was kept constant. The results are shown in Table 1.

Table 1. *The influence of concentration of substrate on apparent potency of purified hyaluronidase*

(*a*) Purified testis enzyme

	Dilution of enzyme						
	1 : 500	1 : 1000	1 : 2000	1 : 4000	1 : 8000	1 : 16,000	1 : 32,000
Double conc. substrate	+	+	±	0	0	0	0
Usual conc. substrate	+	+	+	+	±	0	0
Half conc. substrate	+	+	+	+	+	±	0

(*b*) Purified *Cl. welchii* enzyme

	Dilution of enzyme						
	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200
Double conc. substrate	+	+	+	0	0	0	0
Usual conc. substrate	+	+	+	+	±	0	0
Half conc. substrate	+	+	+	+	+	+	0

\+ Complete reaction; no clot or threads. ± Partial reaction; threads only. 0 No reaction; unmodified clot.

It will be seen that, within the limits of accuracy of this particular test, which was done at only two-fold dilutions of the enzyme, the apparent potency of both enzymes is inversely proportional to the concentration of the substrate mixture. This confirms the observation of Robertson *et al.* [1940] as to the influence of substrate concentration upon this manifestation of enzymic activity, and contrasts with the results obtained by Madinaveitia & Quibell [1940] and McClean & Hale [1941], using a viscosimetric technique; the latter workers reported that the reaction constant and the half-life time of the viscous substrate are constant for a fixed amount of enzyme, and independent of the concentration of the substrate, which indicates that, although the time taken to reach half-viscosity level remains constant, the time taken to carry the reaction to completion increases proportionally to the concentration of the substrate.

Rate of the reaction

Table 2 shows the result of a preliminary experiment in which two streptococcal and two testicular enzyme preparations were titrated after incubation periods varying from 5 to 80 min. The diluted enzyme solutions were warmed for 5 min. in the bath at 37° before the addition of previously warmed substrate mixture. At the end of the period fixed for incubation the racks were transferred to ice-cold water. These tests showed that the reaction was still proceeding rapidly at 20 min. and, in fact, that there was no noticeable lag even after 80 min. It seemed, therefore, that the empirically selected incubation time of 20 min. satisfies the demand that results should be read at a time when the reaction is still proceeding at the initial velocity. This period has the further advantage that it is the same as that used when assaying enzymes viscosimetrically and by diffusion in the skin.

Table 2. *The influence of the period of incubation on the apparent titre of testicular and streptococcal hyaluronidase*

The figures indicate the highest dilution of enzyme which prevented the clotting of the substrate.

Period of incubation min.	Source of enzyme (dilution): *Streptococcus* C 21 whole filtrate	Testis	*Streptococcus* C 7 purified	Testis
5	1 : 320	1 : 10	1 : 200	1 : 1,000
10	1 : 320	1 : 20	1 : 400	1 : 2,000
20	1 : 640	1 : 20	1 : 400	1 : 4,000
30	1 : 1280	1 : 40	—	—
40	1 : 1280	1 : 40	1 : 800	1 : 16,000
60	1 : 2560	1 : 80	—	—
80	—	—	1 : 1600	1 : 64,000

A series of observations were made in which, after preliminary titration, 5 ml. of the appropriate enzyme dilution were mixed with 10 ml. of substrate mixture, both warmed before mixing, and samples of this mixture examined for clotting power at regular intervals. Four dilutions of each enzyme were tested in this way. In addition to the streptococcal and testicular enzyme preparations previously tested, a crude *Cl. welchii* culture filtrate and a purified *Cl. welchii* enzyme were thus examined (see Table 3).

It will be seen that the time taken to destroy the clotting power of the substrate is approximately proportional to the dilution of the enzyme, and this was true for all the enzymes examined. This method of titrating the enzymes did not appear to be as convenient, or as accurate, as that depending upon the determination of the highest effective dilution after a fixed period of incubation; the taking of samples is very time-consuming and, moreover, there is a variable period in which clotting power is partially destroyed, as evidenced by the appearance of threads in the mixture. It is sometimes difficult to determine exactly when there are no threads present. For these reasons the fixed period of 20 min. incubation is preferred to the method of determining the time taken by one dilution of enzyme, which was used by Robertson *et al.* [1940].

Table 3. *The relation between the dilution of the enzyme and the time taken to destroy the clotting power of the substrate mixture at 37°*

The times shown in this table are those from the time of mixing enzyme and substrate to that at which the failure to form a typical mucin clot was noticed. Complete destruction of clotting power was preceded by a variable period in which threads appeared in the substrate mixture.

Purified testicular enzyme: Dilution of enzyme	Threads only (time min.)	No clot or threads (time min.)	*Streptococcus* C7 culture-filtrate: Dilution of enzyme	Threads only (time min.)	No clot or threads (time min.)
1 : 2,000	5	7	1 : 200	4	6 or 10
1 : 4,000	—	11	1 : 400	12	16
1 : 8,000	20	22	1 : 800	30	32
1 : 16,000	35	45	1 : 1600	55–60	60–55

Influence of temperature on the reaction

Robertson *et al.* [1940] observed the influence of temperature on the rate at which a fixed weight of purified mucin was broken down by the enzyme obtained from *Cl. welchii*. They concluded that the rate of the reaction increased in proportion to the

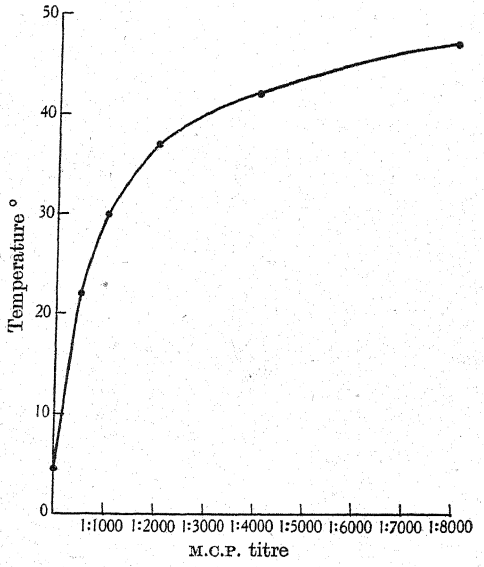

Fig. 1. Influence of temperature on the activity of purified testicular hyaluronidase.

increase of temperature from 0 to 40°, and that above 50° destruction of the enzyme took place rapidly. The figure obtained by them for K_{10} was 1·75.

A sample of purified testicular hyaluronidase was titrated by the routine M.C.P. test at various temperatures, the period of incubation for all these tests being 20 min. The results are shown in Fig. 1. In addition to these results, a test lasting 40 min. was carried out at 22° and one lasting 1 hr. 50 min. at 4°. It will be seen that there was no activity detectable during 1 hr. 50 min. at 4°, but that the apparent titre of the enzyme increased rapidly as the temperature was raised. The rate of this increase was greater at the higher temperature than would have been necessary to give a figure of about 2 for K_{10}. There was no evidence of destruction of the substrate-protein mixture alone at the higher temperatures.

Influence of concentration of salt on the reaction

In the M.C.P. test already described the solvent is distilled water, except for the albumen portion of the substrate-protein mixture. Thus the final concentration of NaCl in the system is only 0·024*M*. It was desirable to determine the effect of increasing the salt concentration on the activity of the enzyme and the kinetics of the reaction. A preliminary experiment showed that, in the absence of enzyme, the substrate-protein mixture would not form a typical mucin clot if the concentration of salt was raised above 0·11*M*. A series of titrations of a purified testicular enzyme were then carried out, in which the NaCl concentration was raised from 0·0024 to 0·1*M*, with the result shown in Table 4. The trace of NaCl present in the lowest concentration was due to the salt present in the 10 % stock albumen solution from which test solutions were made.

Table 4. *The influence of NaCl concentration on the M.C.P. titre of testicular hyaluronidase*

Conc. of NaCl *M*	Highest effective dilution of enzyme
0·0024	<1 : 500
0·017	1 : 1,000
0·033	1 : 4,000
0·07	?1 : 16,000
0·11	?1 : 32,000

The activity of the enzyme increases with salt concentration up to at least 0·1*M*, but the determination of the end-point is obscured above 0·033*M* by the development of a flocculent precipitate, on the addition of acetic acid, in those tubes containing the higher dilutions of enzyme. It appears, therefore, that the salt concentration used in the routine test (0·024*M*) is very near that at which maximum activity of the enzyme combined with a clearly defined end-point is obtained. Robertson *et al.* [1940] have already reported that the enzyme from *Cl. welchii* is inactive in the absence of salt, and that the activity increases as the concentration of various salts in the system is raised to 0·08*M*. It appears from the work of McClean & Hale [1941] and Madinaveitia & Quibell [1941], using a viscosimetric technique, that the activity of the enzyme increases up to an optimum concentration of salt, which varies for the different enzymes examined from 0·1 to 0·3*M*. A further increase of salt causes a rapid fall in activity. Madinaveitia & Quibell further pointed out, that if the concentration of NaCl is reduced below 0·1*M* the reaction time is no longer inversely proportional to the concentration of enzyme.

Influence of pH on the reaction

In the routine M.C.P. test, as described, no buffer is added and the reaction is carried out at approximately *p*H 7·0. Preliminary tests showed that McIlvaine's citric acid-phosphate buffer solutions could not be used in a concentration stronger than 0·5*M* without themselves inhibiting the clotting power of the substrate mixture. When diluted enzyme solutions are added to the system containing 0·5*M* buffer, over a range from *p*H 4·4 to 8·0, it was found that the end-point of activity was obscured by the development of a flocculent precipitate. Attempts to overcome this difficulty by the use of other buffer systems have been unsuccessful for the same reason and, in addition, the apparent titre of the enzyme at the same *p*H may vary owing to the inhibiting action of the ions in some of the systems employed. Control of the *p*H by the use of 0·1*N* HCl and NaOH was unsuccessful, since the substrate mixture clotted spontaneously if the *p*H was reduced below 5·8.

Limits of accuracy of the M.C.P. test at various dilution levels

Observations were made to determine the standard error of this test at different dilution levels. Repeated tests were made on the same purified and dried testicular enzyme in twofold dilutions, and at 50 and 20 % dilution differences. The test in twofold dilutions was repeated 19 times and gave a constant end-point of 1/8000 with no detectable variation. When it was repeated 15 times at 50 % differences in dilution, an end-point of 1/9000 was obtained in 11 tests, of 1/6000 in 2 tests and of 1/13,500 in 2 tests. The standard error at this level is therefore approximately 25 %. The actual reading of the tests at this level is slightly more difficult than at twofold dilutions, since one or more tubes containing dilutions near the end-point have to be very carefully inspected to exclude the presence of a thread of

precipitate. The tests at 20 % differences present the same difficulty in the determination of the end-point, and moreover, occasionally a tube containing a dilution of enzyme below the apparent end-point may show a trace of precipitate. The tests at this level, were, however, repeated 18 times with the following result:

No. of tests	End-point (dilution)	Difference from mean (%)
11	1/8400	—
4	1/6910	20
2	1/10,000	20
1	1/4800	40

Seven out of 18 tests had a doubtful tube below the apparent end-point. The standard error of this test is 18 %.

If the results obtained at the 50 and 20 % dilution levels are combined we may conclude that the standard error of the test as done here is approximately 22 %, and therefore about two-thirds of the results will be within 22 % of the true value, and 20 out of 21 will be within 44 % of this.

When a probit/log-dose curve was fitted to the results from the 18 tests at 20 % dilution differences, by taking the 18 tubes at any one dilution as a group with a percentage response, it was found that the departure from linearity was significant in that higher dilutions gave a much steeper slope than lower ones. From this, it is clear that if, for example, the test were repeated 6 times on small groups of tubes, and the correct end-point taken as that of 50 % of the tests with readings restricted to below 60 %, the method of assay would be very delicate indeed.

Substrate solutions showing any evidence of loss of viscosity (as described under 'Methods', p. 170), were not used for this test, and standard preparations of the substrate and of the albumen were kept in the dry state for comparative tests. Under these conditions there was no evidence of variation in the sensitivity of the substrate mixture. Systematic observations have not been made to determine whether any change in the apparent titre of enzymes is found, when these are retested after varying intervals of time, but chance observations on some of the dried purified enzymes tested at intervals up to 3 months do not indicate any significant variation. I am indebted to Dr C. W. Emmens of the National Institute for Medical Research, Hampstead, for the statistical examination of these results and for much valuable advice.

The neutralization of the M.C.P. reaction by anti-sera

The spread of bacterial diffusing factors in the skin can be inhibited by sera from rabbits immunized with purified diffusing factors, which are free from detectable bacterial toxin [McClean, 1936]. The anti-sera used contained no detectable antitoxin. The serum prepared against the diffusing factor obtained from *Cl. welchii* inhibited the spread of the homologous factor, but not that of the factor obtained from *Vibrion septique* (*Cl. septicum*), and *vice versa*. More recently [McClean & Hale, 1941] it was confirmed that these sera also inhibit the viscosity-reducing, and hydrolytic, activity of the hyaluronidase obtained from the homologous organisms. It was of interest to investigate the influence of these sera on the M.C.P. activity of these enzymes, and to study the activity of sera prepared against enzymes from other sources.

Technique. A fixed dilution of the anti-serum was tested against increasing dilutions of the enzyme. This was found to give a clearer end-point than the reverse method of titrating increasing dilutions of serum against a fixed dose of enzyme.

Since the serum dilutions (1 : 10) were made with physiological saline the substrate-protein mixture was made up entirely with distilled water, in order to keep the salt content of the system the same as that of the routine M.C.P. test. Enzyme solutions were made at twice the concentration to be tested and then mixed with an equal volume of the diluted serum. Thus in the final mixture there was a constant 1 : 20 dilution of serum, mixed with increasing dilution of enzyme. The enzyme-serum mixtures were left at room temperature for 30 min. before the addition of the substrate-protein mixture and subsequently incubated at 37°.

In each test controls were set up in which the enzyme was titrated in the absence of any serum, and also in the presence of correspondingly dilute normal serum. The activity of the sera was expressed as the number of minimal clot-preventing doses of enzyme which would be neutralized by the undiluted serum.

Gas gangrene anti-hyaluronidase sera. The sera prepared against the diffusing factor, which had been shown [McClean, 1936; McClean & Hale, 1941] to neutralize the diffusing, viscosity-reducing and hydrolytic activity of the extracts of *Cl. welchii* and *Vibrion septique*, were tested by the M.C.P. method. Of the two *Cl. welchii* anti-sera, one neutralized from 80 to 160 minimal M.C.P. doses of the homologous enzyme, and the other 160 to 200 doses. Of the two *Vibrion septique* anti-sera, one neutralized more than 100 doses of enzyme, and the other between 40 and 100 doses. It was not possible to test these *Vibrion septique* sera more accurately as no sufficiently potent enzyme from this source was available.

There was no cross-neutralization of the *Cl. welchii* enzyme by *Vibrion septique* anti-serum or *vice versa*.

Streptococcal anti-hyaluronidase sera. Groups of six rabbits were immunized with purified enzyme

obtained from group A type 4 and group C type 7 streptococci. The culture filtrates were purified by adsorption on to alumina Cγ and elution with Na_2HPO_4 solution according to the method described by Madinaveitia [1939]. One of these crude filtrates, and the corresponding purified preparation, was kindly examined by Dr E. W. Todd, of the L.C.C. Antitoxin Laboratories for streptolysin O. He reported that, whereas the crude filtrate contained considerable quantities of this haemolysin, none was detectable in the purified preparation. The group A and group C anti-sera were also tested by Dr Todd for antitoxin both before and after immunization. Unfortunately, the tests on the group C anti-sera did not give completely clear-cut results owing to deterioration of the sera during an interval elapsing before they were tested. There was, however, evidence of a slight increase in anti-haemolysin titre after the second course of injections over the figure to be expected for a similar group of normal rabbits. This result suggests that there may have been a small amount of haemolysin toxoid in the purified enzyme preparation. The sera from the group A rabbits all showed less than 1 unit of anti-streptolysin O both before and after immunization. These results indicate that the group A purified enzyme preparations did not contain significant amounts of streptolysin O or the corresponding toxoid, and that their anti-hyaluronidase activity could not be ascribed to any increase in anti-haemolysin.

Tests for anti-hyaluronidase activity with the sera from the six rabbits immunized with the group C enzyme showed that after the first course of 7 injections they neutralized from 160 to 500 minimal M.C.P. doses of the homologous enzyme; after the second course of 4 injections the sera from all these rabbits neutralized 750 or more doses of enzyme. The sera obtained from the animals immunized with the group A enzyme neutralized from 40 to 160 doses of enzyme after the first course of injection. Cross-neutralization tests showed that there is no neutralization of group A enzymes by group C sera or *vice versa*. On the other hand, neutralization tests carried out with enzymes from group C types 20 and 21 strains and the serum prepared against the enzyme derived from group C type 7 strain showed that this serum was equally potent against these enzymes. It appears, therefore, that in their present state of purification, these streptococcal enzymes are group- but not type-specific.

Testicular anti-hyaluronidase sera. Through the courtesy of Dr Ungar of the Glaxo Laboratories a sample of rabbit serum, prepared against partially purified enzyme from bull's testis, was obtained. This serum had been shown by Dr Ungar and his colleagues to inhibit the spreading activity in the skin of the homologous testicular enzyme (private communication). Neutralization tests by the M.C.P. method were set up with this, against a purified enzyme derived from bull's testis, a crude mouse testis enzyme, and bacterial enzymes derived from *Cl. welchii*, *Vibrion septique*, group A and group C streptococci. This serum neutralized 200 minimal M.C.P. doses of the homologous bull's testicular enzyme, but showed no neutralizing activity against the enzyme from mouse testis nor any of the bacterial enzymes. This anti-testicular serum was also tested viscosimetrically with the homologous enzyme. A 1 : 10 dilution of the serum neutralized the activity of at least 7 viscosity-reducing units of this enzyme.

It should be borne in mind that the apparent specificity of these anti-hyaluronidase sera, prepared from various bacterial enzymes and from testis, may be due to the partial purification of the enzymes used as antigens, and to the fact that these enzymes were still linked to some other bacterial or tissue component, which determined the specificity of the anti-sera.

The relation between the M.C.P. reaction and viscosity reduction by the enzyme

It was known that the highest dilution of a purified testicular enzyme which would destroy the clotting power of a substrate protein mixture in 20 min. would reduce the relative viscosity of the substrate only from 153 to 148 in that time. The dilution of this sample was 1 : 100. When this sample of enzyme was tested viscosimetrically in a system containing the same amount of NaCl as the M.C.P. system, it was found to contain 16 viscosity-reducing units (V.R.U.); by definition [McClean & Hale, 1941], 1 V.R.U. is that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level halfway between its original figure and that of the solvent employed in 20 min. Thus the clotting power of the substrate mixture was destroyed in 20 min. by a dilution of enzyme containing only 0·16 V.R.U. It was of interest to determine how rapidly a solution containing 1 V.R.U. would prevent the formation of the clot.

When the substrate mixture and a dilution of enzyme containing 1 V.R.U. were warmed separately to 37°, the capacity to form a typical mucin clot was destroyed in less than 1 min., threads only developing in a sample tested after 1 min. A 2 min. sample remained clear after the addition of acetic acid. These observations indicate that mucin-clot prevention is the first stage in the degradation of the substrate, and that this change can be detected before any appreciable fall in viscosity occurs. The fact that diffusion in the tissues can also be detected immediately, and occurs in high dilution

suggests that this phenomenon may be closely related to this primary stage in the degradation of the substrate.

The combined influence of salt concentration and of pH on the viscosity-reducing activity of the enzyme

It has been shown [McClean & Hale, 1941; Madinaveitia & Quibell, 1941] that the concentration of salt profoundly affects the viscosity-reducing activity of these enzymes and that this effect varies with enzymes from different sources and with the nature of the ions present. The combined effect of salt concentration and *p*H was studied. Madinaveitia *et al.* [1940] reported their failure to obtain correlation between skin-diffusing and viscosity-reducing activities of several enzymes from different sources. Their viscosity estimations were, however, carried out at *p*H 4·6 in a final concentration of *M*/6 buffer solution, whereas the diffusion in the skin presumably took place at about *p*H 7·0 in isotonic salt concentration.

Two samples of testicular enzymes purified by different methods, one purified *Cl. welchii* enzyme and one crude *Cl. welchii* culture filtrate, were titrated viscosimetrically in McIlvaine's citric acid-phosphate buffer at *p*H 4·6 and 7·0. The titrations were carried out in duplicate with final concentrations of *M*/6 and *M*/60 buffer in the system. It had already been established that the apparent titre of the enzyme was not materially affected by substituting McIlvaine's buffer at *p*H 4·6 for the Na citrate usually employed. The results obtained in these titrations are shown in Table 5.

Table 5. *The combined influence of salt concentration and of pH on the viscosity-reducing activity of the enzyme*

	At *p*H 7·0		At *p*H 4·6	
Source of enzyme	Buffer *M*/60 V.R.U.	Buffer *M*/6 V.R.U.	Buffer *M*/60 V.R.U.	Buffer *M*/6 V.R.U.
Testis Gl. 3	1,280	670	205	830
Testis P.M. 1	125	37	17·6	47
Cl. welchii 74 (purified)	15,150	4000	4000	6100
Cl. welchii (filtrate)	50	15·5	27	27

Note. McIlvaine's citric acid-phosphate buffer used throughout.

V.R.U. viscosity reducing units.

It will be seen that, with all three purified enzymes, there is a marked decrease in the apparent activity at *p*H 7·0 if the concentration of buffer is increased from *M*/60 to *M*/6. On the other hand, at *p*H 4·6 there is a substantial increase in activity as the concentration of buffer is increased. Thus, if titrations of any of these enzymes were carried out at different *p*H's in the presence of *M*/6 buffer it would appear that their activity would be greater at *p*H 4·6 than at *p*H 7·0. If, however, the tests were done in *M*/60 buffer the enzymes would be much more active at *p*H 7·0 than at *p*H 4·6. The crude *Cl. welchii* enzyme shows a similar response

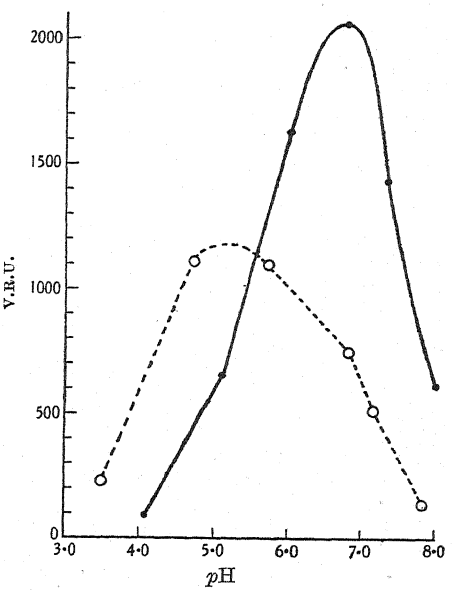

Fig. 2. Combined influence of *p*H and salt concentration. Purified testicular hyaluronidase. •—• titration in *M*/60 buffer. o---o titration in *M*/6 buffer. V.R.U. viscosity-reducing unit.

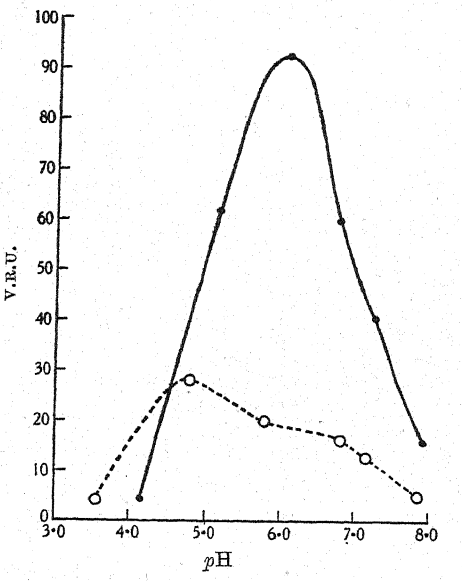

Fig. 3. Combined influence of *p*H and salt concentration. Crude *Cl. welchii* enzyme. •—• titration in *M*/60 buffer. o---o titration in *M*/6 buffer. V.R.U. viscosity-reducing unit.

to increased concentration of buffer at *p*H 7·0 but no change in titre at *p*H 4·6. The reason for this is clear from Fig. 2.

Curves showing variation of activity with change of *p*H were obtained for one of the purified testicular enzymes and for the crude *Cl. welchii* enzyme in both *M*/6 and *M*/60 buffer solutions. The *p*H of the systems in which the observations were made were checked electrometrically. Fig. 2 shows that, in a buffer concentration of *M*/60 the *p*H optimum of the testicular enzyme is about 6·8, whereas in *M*/6 buffer the optimum shifts to about *p*H 5·0. Moreover, around the *p*H optimum the activity of the enzyme is very much greater in the more dilute buffer. Fig. 3 shows a similar picture for the crude *Cl. welchii* enzyme though the shift of *p*H optimum as the buffer concentration is altered is not as marked as it is for the testicular enzyme; in *M*/60 buffer the optimum activity is obtained at about *p*H 6·0 and in *M*/6 buffer at about *p*H 4·5. This figure shows why the activity of this enzyme at *p*H 4·6 appeared to be the same in both concentrations of buffer; this is the point at which the two curves cross and the curve of activity in *M*/60 buffer is exceedingly steep.

These results cast doubt on the validity of the '*p*H optima' for this group of enzymes that have been previously reported by us and many other workers. It is obvious that the *p*H curves are profoundly modified not only by the concentration of the salts that are present but also by the nature of the ions in the system [McClean & Hale, 1941; Madinaveitia & Quibell, 1941]. M. G. Macfarlane & H. J. Rogers state (private communication) that with different buffer systems the *p*H optimum of the lecithinase derived from *Cl. welchii* is different. Similarly, Myrbäck [1926] and Sherman, Caldwell & Adams [1928] found that some ions increase the activity of amylases and change the *p*H optimum.

The correlation between different methods of assaying enzymic activity

Nine representative enzyme preparations obtained from different sources and showing a wide range of activity were selected for assay, by their diffusion in the skin, their viscosity-reducing activity and by the mucin-clot prevention test. A complete description of the test for diffusing activity is given by J. H. Humphrey [1943].

Table 6. *Tests of enzymic activity by different methods*

Source of enzyme	Diffusing activity (Glaxo units/ml.)	Viscosity reducing activity units	Mucin-clot prevention. Highest effective dilution	Ratios: Diffusion / V.R.U.	Ratios: Diffusion / M.C.P.	Ratios: M.C.P. / V.R.U.
Streptococcus C 7 (purified)	750	12	208	63	3·6	17·3
Glaxo standard prep. (1 mg./ml.)	2,000	29	100	69	20	3·5
Cl. welchii (purified)	4,000	57	860	70	4·75	15·1
Cl. welchii (filtrate)	8,000	57	750	140	10·6	13·2
Testis (P.M. 1)	8,000	125	1,040	64	7·7	8·3
Streptococcus (A 4 filtrate)	10,000	93	1,040	107	9·6	11·2
Streptococcus (C 7 filtrate)	10,000	225	10,000	44·5	1·0	44·5
Testis (G 3)	200,000	1,280	8,000	156	25	6·2
Cl. welchii (W 74 purified)	800,000	15,150	125,000	52	6·4	8·3
		Coefficient of correlation		0·980	0·978	0·998
				$P \ll 0·001$	$P \ll 0·001$	$P \ll 0·001$

Diffusing activity. Tested in twofold dilutions; see Humphrey (1943). 0·5μg. of the Glaxo standard preparation contains 1 diffusing unit.

Viscosity-reducing activity. Estimated at *p*H 7·0 in *M*/60 McIlvaine's buffer solution. Unit defined in text. Results accurate to ±10%.

Mucin-clot prevention. Titrated at 20% differences in dilution.

In the light of the observations described in the foregoing section, it was decided to carry out viscosimetric titrations in *M*/60 buffer at *p*H 7·0. The error of this method has been shown to be less than ±10%. The M.C.P. test was carried out at 20% differences in dilution, and the standard error of this test is approximately 22% (see Table 6). It will be seen that the coefficient of correlation between any two of the three tests is very high, and that the ratio between the individual values for any sample obtained by the skin diffusion, and by the viscosimetric tests, are as good as could be expected, when the limit of accuracy of the former test is taken into consideration. When the ratios between the values obtained by the M.C.P. test, and by either of the other methods, are examined, the result is not so satisfactory. The variation is much larger than can be explained by the internal errors of the different types of test. This difference in the ratios does not show any constant trend as the potency of the enzymes increase, nor does it vary consistently with the source from which the enzyme was obtained. At this stage it is not possible to give any satisfactory explanations for this discrepancy, but the fact that the coefficient of correlation for all three tests, on nine different enzymes from

diverse sources and of a wide range of activity, is so high, is very strong evidence that all three tests are, in fact, measuring the activity of the same agent. In view of the contradictory reports that have appeared in the literature it is interesting to note, that when the viscosimetric assay is carried out at *p*H 7·0, in approximately physiological salt concentration, very good agreement is obtained with the results of skin-diffusing activity.

SUMMARY

1. A method of assaying hyaluronidase is described, which depends upon the destruction by the enzyme of the power of a substrate-protein complex to form a typical 'mucin clot' on the addition of acetic acid.

2. It appears that the destruction of the clotting power of the substrate is an early stage in its degradation, which can be detected before any appreciable fall in viscosity occurs.

3. Sera which inhibit the diffusing and viscosity-reducing activity of these enzymes also inhibit the mucin-clot prevention reaction. Sera prepared against enzymes obtained from *Cl. welchii* and *Vibrion septique* are species- but not type-specific. Those obtained against streptococcal enzymes are group- but not type-specific. A serum prepared against diffusing factor from bull's testis inhibited this enzyme, but did not inhibit a testicular enzyme from the mouse, or any of the bacterial enzymes.

4. Both the apparent *p*H optimum of viscosity-reducing activity, and the titre of the enzyme, are markedly affected by the concentration and nature of salts in the system.

5. Nine enzymes from various sources have been examined for diffusing activity in the skin, by viscosimetry and by the mucin-clot prevention test. The conclusion is drawn that all three tests measure the activity of the same agent.

I wish to express my appreciation of the keen cooperation and many constructive suggestions of Mr C. W. Hale.

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Studies on Diffusing Factors

3. A NEW BIOLOGICAL ASSAY OF DIFFUSING FACTOR IN GUINEA-PIGS

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(*Received 13 November 1942*)

The most accurate biological method of assaying diffusing factor hitherto published is that of Bacharach, Chance & Middleton [1940]. This method compares the activities of pairs of preparations by measurement of the mean bleb size produced in suitable rabbits, using a 'four-point' assay. Since the log dose-response curve is sigmoid these authors found that the four doses must be carefully adjusted so as to lie upon the straight portion of the curve, but if such precautions are observed they claim to be able to distinguish satisfactorily between doses in the ratio of 2 to 1. In the course of work undertaken to establish the identity, or otherwise, of diffusing factor and hyaluronidase, it became desirable to be able to detect twofold differences in diffusing-factor activity. In view, however, of the difficulty caused by the non-linearity of the log dose-response curve [Evans & Madinaveitia, 1940], it was decided, at the suggestion of Mr C. W. Hale, to devise an accurate assay of the least dose which would produce a significant increase in bleb area in a given time, when injected intracutaneously

into a group of guinea-pigs. The principle is the same as that underlying tests of minimal lethal dose, etc. The time chosen, which is the shortest convenient, is also such that active diffusion is still taking place when the bleb size is measured, and the measurement is made before the initial velocity of reaction has fallen significantly. The method to be described has provided, in our experience, an absolute assay, inasmuch as the minimal diffusing dose is the same for repeated assays of the same preparation, using different batches of animals. With reasonable precautions in the choice of animals, and within the limits of accuracy of the test, it should be possible for different workers to use comparable units without necessarily sharing a common standard preparation.

METHODS

Procedure for assay

General. Two control and 6 test doses of diffusing factor (D.F.) are injected intracutaneously into each of 6 guinea-pigs, with haemoglobin as an indicator of the bleb size [Madinaveitia, 1938]. After exactly 20 min. each animal is killed and the skin of the back is rapidly flayed. The areas of the blebs are measured on the underside of the skin, and the minimal diffusing dose is taken as the least amount of D.F. which will produce a 20% increase in mean area as compared with the mean of the controls.

Animals. The guinea-pigs should weigh over 300 g. and their skins should be white. For each assay 7 are required, and their backs are clipped before use.

Solutions. The dilutions of D.F. are made in physiological saline shortly before injection. To each dilution is added an equal volume of horse haemoglobin solution, made by washing horse red cells with physiological saline six or seven times on the centrifuge, laking by the addition of distilled water up to the original volume of the blood, and making up to 0·85% (w/v) NaCl by the addition of solid NaCl, in quantity calculated from the chloride content of the solution. The final dilution of D.F. is thus 1 : 2 of the original. It is important to wash the cells very well, since horse serum inhibits the action of D.F. and it is best to add the haemoglobin solution after making the dilutions, since it is viscous and renders accurate pipetting difficult.

Injections. 0·2 ml. of each dilution, and two controls of 0·2 ml. of equal parts haemoglobin solution and saline, are injected intracutaneously with Nimrod syringes into each guinea-pig. The position on the guinea-pig does not matter (see below), but the order should be kept constant to facilitate reading later. A separate syringe should be used for each dilution. It is convenient to inject one guinea-pig every 3 min., since the first is then ready for killing and measurement soon after the last one has been injected. There is a small leak back from each bleb, but under standard conditions this is fairly constant in amount.

Measurement of blebs. Exactly 20 min. after injection each animal is killed by a blow on the head, and the skin containing the blebs is removed from its back. The skin is then laid out flat with the inside uppermost and sponged with cold water to clean the skin and cause the muscular layer to relax. The blebs stand out sharply, and are elliptical, except for those in which very considerable diffusion has occurred. The area is obtained by measuring the shortest and the longest diameters with dividers and multiplying their product by $\frac{1}{4}\pi$. Since the time taken for measurements on each guinea-pig is 3 min. or less the time-table can be adhered to fairly accurately.

A preliminary orientating assay is made in a single guinea-pig using six tenfold dilutions of D.F. Having found roughly what dose gives a 20% increase in bleb area over the controls, the accurate assay is carried out using six twofold dilutions of D.F. adjusted to cover the end-point arrived at in the preliminary assay. Examples are given in Table 3.

RESULTS

The test described above was worked out as the result of an experiment in which each of 6 guinea-pigs was given 8 intracutaneous doses of 0·2 ml. haemoglobin-saline in a constant order, and bleb area measured in mm.² after 20 min. (Table 1).

Table 1. *Variation in area of blebs in control experiments without diffusing factor*

	Animal no.					
	I	II	III	IV	V	VI
	Size of individual blebs (mm.²)					
	159	165	195	163	170	206
	180	164	187	200	176	214
	180	143	181	204	201	212
	170	162	188	159	177	214
	176	177	195	189	—	187
	177	174	201	176	181	176
	153	194	176	179	189	201
	167	194	174	193	195	—
Means	170	172	187	183	184	201
Greatest deviation from mean as % of mean: Plus	6	13	7·5	11·5	9	6·5
Minus	10	17	7	13	7·5	12·5
Coefficient of variation	5·5	9·3	4·5	8·4	4·6	6·8

From the results in Table 1 it can be seen that although most of the guinea-pigs have a similar mean bleb size, occasionally one differs from the others. The size of individual blebs on any one guinea-pig was fairly constant, and appeared to grow more constant as our skill improved in later tests. There is no constant variation with position on the animal. If a variation equal to twice the standard deviation is considered significant, it appears that in each animal a 20% increase in bleb size, compared with the mean for that animal, would be significant. In our test by taking 2 blebs on each of 6 guinea-pigs as a control we have 12 control blebs against which to compare 6 test blebs for each dose level.

A similar experiment to the above was done with a known diffusing dose, to ascertain whether the same degree of constancy could be obtained when diffusion was occurring. One minimal diffusing dose of preparation G 3 was used throughout. This, in

previous assays at this dose level, had given mean percentage increases in area of 20 and 25 %. In this test the size of the control blebs showed fair constancy and there was fairly good agreement between test doses on any one guinea-pig—apart from one bleb in each of animals IV and V, which differ markedly from the others, and which increase considerably the coefficient of variation. There was definite variation in sensitivity from animal to animal, but the average amount of diffusion for all pigs at this dose level (28 %) agrees well with previous assays of the same preparation (Table 2).

In Table 3 is given an example of two separate complete assays of a testicular diffusing-factor preparation, which can be detected by our test at a dilution of 1 : 40,000. It will be seen that in the critical range the agreement of the mean values is quite good, despite considerable variation in individuals.

In the first 10 tests which we made, comprising a total of 60 guinea-pigs, an analysis was made of the individual responses as compared with the averaged response of each group of 6 animals. The results (Table 4) showed that 50 out of 60 animals gave an end-point within one twofold dilution on either side of that dilution which was the end-point deduced from the mean response of the batch.

Table 2. *Variation in area of blebs with one minimal diffusing dose of diffusing factor*

	Animal no. I	II	III	IV	V	Combined totals
	Bleb areas (mm.²)					
	240	214	320	269	276	
	254	213	290	276	254	
	262	227	252	(330)	268	Mean test bleb 257
	254	213	267	254	240	
	248	212	310	254	(203)	
	230	228	266	274	314	
Control blebs	198	188	200	201	200	Mean control bleb 201
	212	200	214	200	200	
Mean percentage increase over controls	21	12	37	38	29	28
Coefficient of variation for test blebs	4·3%	3%	8·9%	9·8%	13·6%	For all test blebs 12·7%

Table 3. *Data for two separate assays on the same preparation of diffusing factor*

(*a*) Preliminary assay of diffusing factor on single guinea-pig

Dilution of D.F.	Bleb area mm.²	Increase over controls %	Dilution of D.F.	Bleb area mm.²	Increase over controls %
1 : 10^2	475	166	1 : 10^6	189	6
1 : 10^3	314	76	1 : 10^7	201	13
1 : 10^4	219	23*	Control	175 } 179	
1 : 10^5	206	16	Control	182 }	

(*b*) Complete test on groups of guinea-pigs

	First test						Second test					
Animal no. ...	1	2	3	4	5	6	7	8	9	10	11	12
Dilution of D.F.	Bleb areas (mm.²)											
1 : 5,000	227	297	314	329	304	348	277	269	294	245	410	307
1 : 10,000	249	262	260	262	227*	284	255	283*	232	252	314	226
1 : 20,000	254*	255	262	255	189	—	213	176	255	214	283*	280
1 : 40,000	212	259*	258*	269*	187	299	245*	176	218*	214*	190	208*
1 : 80,000	182	171	176	170	213	299*	189	187	176	165	185	177
1 : 160,000	224	188	201	201	205	227	165	201	149	182	171	177
Control	192	232	173	163	194	205	176	165	182	153	180	189
Control	176	212	200	154	—	214	160	156	143	171	189	154

* Greatest dilution showing 20 % increase in area over controls.

(*c*) Summary: Mean for six guinea-pigs

	First test	Second test		First test	Second test
Controls	192	167			
1 : 160,000	208 (plus 8%)	174 (plus 4%)	1 : 20,000	233 (plus 21%)	237 (plus 42%)
1 : 80,000	202 (plus 5%)	180 (plus 8%)	1 : 10,000	258 (plus 35%)	260 (plus 56%)
1 : 40,000	230 (plus 20%)	209 (plus 25%)	1 : 5,000	305 (plus 59%)	300 (plus 80%)

Table 4. *Scatter of assay results with 60 animals*

Assigned dilution	No. of animals giving end-point at: 1 dilution		2 dilutions		3 dilutions	
	Less	More	Less	More	Less	More
31	11	8	6	2	1	1

With the kind co-operation of Mr Bacharach and his co-workers a series of preparations was assayed by our method, by the method of Bacharach *et al.* [1940], and by a modification of the viscosity-reduction test described by McClean & Hale [1941] which we believe to be accurate to within 10%. The results are listed in Table 5, in which we have

Table 5. *Comparative results with three methods of assay*

Enzyme preparation	Diffusing activity in Glaxo units/ml.*: Our assay	Glaxo assay	Viscosity reducing activity units/ml.
Streptococcus C 7 (purified)	750	4,650	12
Glaxo standard testis (1 mg./ml.)	2,000	(2,000)	29
Cl. welchii (purified)	4,000	4,050	57
Cl. welchii (filtrate)	8,000	4,800	57
Testis (P.M. 1)	8,000	12,400	125
Streptococcus (A 4 filtrate)	10,000	19,600	93
Streptococcus (C 7 filtrate)	10,000	13,500	225
Testis (G. 3)	200,000	19,000	1,280
Cl. welchii (W. 74 purified)	800,000	60,300	15,150

* The conversion of our assay into Glaxo units is carried out as follows: for the Glaxo Standard testis powder, by their definition, one diffusion unit (D.U.) is contained in 0·5 μg. We found on repeated test that a solution of this powder containing 1 mg./ml. showed significant diffusion at a highest dilution of 1 : 400 of the solution. Our minimal diffusing dose (M.D.D.) therefore contains 0·25 μg. and hence 2 M.D.D. corresponds to 1 D.U.

calculated our minimal diffusing doses so as to give the potency of the preparations in terms of the Glaxo Standard, which we assayed by our method. A full discussion of these and other results is given elsewhere [McClean, 1943], from which it will be seen that, within the limits of the tests, there is fair agreement between our minimal diffusing dose assay and the viscosity-reducing activity, whereas with the Glaxo assays the agreement is less good. It is only necessary here to discuss the degree of agreement between the two biological methods of assay. Over the range 2000–10,000 units/ml. this is good, but with the weaker and the stronger preparations the differences are far outside the experimental errors of the two tests.

DISCUSSION

The method of Bacharach *et al.* [1940] is based upon the measurement, in the skins of rabbits, of the total area through which a preparation spreads in a given time, compared with the area of spread of a standard preparation. The areas of diffusion are proportional to the logarithms of the doses, only over a limited range of dilutions, which these workers select for their test. Their method depends upon the evaluation of the slope of the log dose-response curve (b) at that part of the curve at which it is linear, and they state that the values of b, for the standard and for the test blebs, are, in any one experiment, always sufficiently close to permit of accurate calculation. The method described above measures the smallest dose of D.F. which produces significant increase in diffusion, and measures therefore a quantal response. It has in its favour that it is relatively easy to carry out in practice. Statistically, however, it is less satisfactory than the analysis of a continuous variate, as carried out by Bacharach *et al.* [1940]. Yet the results of our assay are in closer agreement than theirs with the determinations of viscosity-reducing activity, when the latter is measured under physiological conditions of salt concentration and pH [McClean, 1943]. Re-examination of all the assays, with a 50% increase in bleb area over the mean controls instead of a 20% increase as the critical response, yielded a similar picture (Table 6). Unlike the method of the Glaxo workers, no standard preparation is included in each test, and therefore analysis requires the construction of a standard log dose-response curve

Table 6. *Comparison of results of assays based on measurements of 20% and of 50% increases in area of bleb*

Enzyme preparation	Viscosity-reducing units/ml.	D.U.* (20% increase)/ml.	D.U./V.R.U.*	D.U.* (50% increase)/ml.	D.U./V.R.U.*
Streptococcus C 7 (purified)	12	750	63	500	42
Standard testis	29	2,000	69	2,000	69
Cl. welchii (eluate)	57	4,000	70	2,000	35
Cl. welchii (crude)	57	8,000	140	2,000	35
Testis P.M.	125	8,000	64	4,000	33
Streptococcus A 4	93	10,000	107	5,000	53
Streptococcus C 7 (crude)	225	10,000	44	10,000	44
Testis G 3	1,280	200,000	156	100,000	77
		Two assays		50,000	38
Cl. welchii 74	15,150	800,000	52	200,000	13

* D.U. Glaxo-diffusing unit. V.R.U. viscosity-reducing unit.

for guinea-pigs under the conditions described. This was attempted, using the figures obtained in all the various assays performed, but the resulting values for the slope of the best straight line (*b*) were so divergent as to render further calculation useless. It is perhaps surprising, in these circumstances, that the method should give results which are apparently more consistent with other properties of the preparations used. We have been unable to arrive at any satisfactory explanation of the difference between the tests, and can only offer a tentative suggestion that, for the biological assay of D.F., the guinea-pig is a more satisfactory animal than the rabbit.

SUMMARY

A method is described for assaying the activity of diffusing-factor preparations in the skin of guinea-pigs, whereby twofold differences in activity can be distinguished. This method, which is based on the determination of the least amount required to produce a 20 % increase in bleb area, gives reasonable agreement with assays of the hyaluronidase content of the same preparations, when this is estimated by the reduction of the viscosity of hyaluronic acid.

I wish to acknowledge the assistance of Mr C. W. Hale who suggested the investigation of this method of test and collaborated throughout.

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A Method for the Estimation of Glucose-1-phosphate and Galactose-1-phosphate in the Liver

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The estimation of glucose-1-phosphate and galactose-1-phosphate in solutions containing only small quantities of inorganic phosphate and reducing substances does not offer any particular difficulties. Both esters are readily hydrolysed by acid and the liberated inorganic P and hexose can easily be determined [Cori & Cori, 1937; Kosterlitz, 1938]. In the liver, however, relatively large quantities of inorganic P (about 30 mg. P/100 g. liver) and considerable quantities of reducing substances (corresponding to 300 mg. glucose/100 g. liver) make it difficult to determine such small quantities of hexose-1-phosphates as may be expected to be present. The acid-soluble organic P fraction obtained from rabbits' livers during galactose assimilation contained only 5–12 mg. galactose or 0·9–2 mg. P/100 g. liver as galactose-1-phosphate and less than 1 mg. glucose or 0·17 mg. P/100 g. liver as glucose-1-phosphate [Kosterlitz, 1939*b*]. Furthermore, the glycogen present in the liver yields glucose on acid hydrolysis and also interferes with the fractionation of the soluble and insoluble Ba salts. For these reasons, a preliminary step in the estimation of the 1-esters in trichloroacetic acid extracts of liver is the removal of the interfering substances, viz. inorganic P, reducing substances, and glycogen.

A description of the method as it has been used for the determination of the hexose-1-phosphates present in the livers of rats fed with glucose or galactose will be followed by a critical examination of the various steps involved in the determination.

METHODS

The excised liver is rapidly washed free from adherent blood, dried between filter paper, and cut into 4–5 pieces which are immediately immersed in liquid oxygen. When frozen, 5 g. or more are weighed and transferred to an ice-cold mortar containing 20 ml. 5 % ice-cold $CCl_3.COOH$ and some acid-washed sand. Throughout the procedure, whenever the reaction is acid, all glass vessels and reagents are kept as near 0° as possible. After the liver has been thoroughly ground, additional 5 % $CCl_3.COOH$ is added in quantity such that each 10 ml. of the final extract represent 1 g. of wet liver. In these experiments the water content of the liver has been assumed to be 80 % of the liver weight. Therefore, a total of 9·2 ml. 5 % $CCl_3.COOH$/1 g.

liver, has been added. The liver and the acid are once more well mixed and allowed to stand for 3–5 min. Then, the pulped tissue and sand are centrifuged down at 3000 r.p.m. for 3 min. To as large a measured sample of the clear supernatant fluid as possible are added one drop of 0·5% alcoholic thymolphthalein solution, a measured quantity of 40% NaOH, and finally of $N/2$ NaOH, drop by drop, until the solution just turns blue. Then for each ml. of the original CCl_3.COOH extract, 0·1 ml. of freshly prepared and filtered 10% mercuric acetate solution is added. After 20 min. standing at 0°, the Hg precipitate is centrifuged off. If the supernatant fluid is not absolutely clear, it is filtered through an acid-washed paper (Whatman no. 541). The excess of mercuric acetate is removed from a measured sample by means of H_2S. The HgS, which has adsorbed the glycogen, is filtered off and washed 4 times with 2 ml. H_2O. The combined filtrate and washings are aerated for 20 min. One drop of 0·5% thymolphthalein solution is added and then finely powdered $Ba(OH)_2$ until the solution just turns blue (about 0·32 g.). The *p*H is adjusted, if necessary, with a drop of 5% CCl_3.COOH or $N/2$ NaOH. 3 vol. redistilled ethanol are added, and the flask stoppered and allowed to stand at 0° for 2 hr. The precipitate is then quantitatively collected in a 50 ml. cup by repeated centrifuging and the flask finally washed out 4 times with 5–10 ml. of 75% ethanol. The precipitate in the centrifuge tube is dried for 15 min. *in vacuo* over $CaCl_2$, and after addition of 2·5 ml. H_2O is well broken up with a fine glass rod; the rod is washed with 0·5 ml. H_2O, and the tube centrifuged for 3 min. at 3000 r.p.m. The supernatant fluid is transferred to another 50 ml. centrifuge tube. This extraction is repeated twice, the supernatant fluids being added to the fluid from the first extraction. 3 vol. ethanol (27 ml.) are then added to the combined extracts, and the centrifuge tube is stoppered and allowed to stand overnight at 0°. After centrifuging, the precipitate is dried *in vacuo* over $CaCl_2$ for 40 min. The precipitate is then dissolved in 5·0 ml. 0·1N HCl; 5·0 ml. 0·4N H_2SO_4 are added and the $BaSO_4$ centrifuged off. The supernatant fluid is transferred to a Pyrex test tube. Inorganic P is estimated in 1 ml. and the reducing power before hydrolysis in 2 ml. 3·75 ml. are transferred to another Pyrex tube, which is stoppered, and hydrolysed at 50° for 30 min. The remainder of the fluid in the original tube, which is fitted with an air condenser, is hydrolysed at 100° for 3 min. Of the hydrolysed solutions 2 ml. were used to estimate increase in reducing power, as previously described [Kosterlitz, 1939*a*], and 1 ml. to estimate increase in inorganic P by a modified Fiske & Subbarow [1925] procedure. The volume of the reaction mixture was 1·17 ml. with P contents from 1 to 12 μg.

Examples:

(1) Liver from a rat fasted for 24 hr.

Liver weight: 7·36 g.	
Sample of CCl_3.COOH extract	63·0 ml.
NaOH	1·52 ml.
10% mercuric acetate	6·3 ml.
Total volume	70·82 ml.

Sample used after centrifuging = 65·0 ml. ≡ 5·79 g. liver. The final precipitate of the organic P was dissolved in 10 ml. 0·25N acid.

1 ml. (≡0·579 g. liver) contained:

Before hydrol.	2·01 μg. inorg. P
After 3 min. hydrol. at 100°	2·70 μg. inorg. P, i.e. an increase of 0·69 μg.
After 30 min. hydrol. at 50°	2·22 μg. inorg. P, i.e. an increase of 0·21 μg.

The increases in P. calculated/100 g. liver were *0·119* mg. and *0·036* mg. (=30·3%) respectively.

The reducing power, calculated as glucose, of 2 ml. (≡1·158 g. liver) was:

Before hydrol.	41·5 μg.
After 3 min. hydrol. at 100°	58·0 μg., i.e. an increase of 16·5 μg.
After 30 min. hydrol. at 50°	47·5 μg., i.e. an increase of 6 μg.

The increases in reducing power calculated as glucose/100 g. liver were *1·43* and *0·52* mg. (=36·4%) respectively. The reducing power before hydrolysis ('initial reduction')/100 g. liver was equivalent to *3·59* mg. 'glucose'.

(2) Liver from a rat fed 1·5 ml. 60% galactose solution/100 g. body weight after a 24 hr. fast, and killed 2 hr. afterwards.

Liver weight: 5·40 g.	
Sample of CCl_3.COOH extract	45·0 ml.
NaOH	1·15 ml.
10% mercuric acetate	4·5 ml.
Total volume	50·65 ml.

Sample used after centrifuging = 45 ml. = 4·00 g. liver. The final precipitate of the organic phosphates was dissolved in 10 ml. 0·25N acid.

1 ml. (≡0·400 g. liver) contained:

Before hydrol.	2·70 μg. inorg. P
After 3 min. hydrol. at 100°	5·36 μg. inorg. P, i.e. an increase of 2·66 μg.
After 30 min. hydrol. at 50°	4·57 μg. inorg. P, i.e. an increase of 1·87 μg.

The increases in P calculated/100 g. liver were *0·665* mg. and *0·468* mg. (=70·3%) respectively

The reducing power, calculated as glucose, of 2 ml. (≡0·800 g. liver) was:

Before hydrol.	90·5 μg.
After 3 min. hydrol. at 100°	125·5 μg, i.e. an increase of 35 μg.
After 30 min. hydrol. at 50°	114·0 μg., i.e. an increase of 23·5 μg.

The increases in reducing power calculated as glucose/100 g. liver were *4·38* and *2·94* mg. (=67·2%) respectively. The initial reduction was *11·3* mg. 'glucose'/100 g. liver.

Certain corrections have to be introduced: for these see the recovery experiments.

The significance of the various values obtained will be discussed later: here the following points may suffice.

The 'initial reduction' is an approximate measure of the quantity of equilibrium ester (hexose-6-phosphate) present. Both glucose-1-phosphate and galactose-1-phosphate are completely hydrolysed by a mixture of 0·05*N* HCl and 0·2*N* H_2SO_4 at 100° in 3 min., while hydrolysis at 50° for 30 min. splits 28·6% of the glucose-1-phosphate present and 77·5% of the galactose-1-phosphate present.

EXAMINATION OF THE METHOD

Acid hydrolysis of glucose-1-phosphate and of galactose-1-phosphate

I. *At 100°.*

(1) Glucose-1-phosphate, prepared according to Hanes [1940] is hydrolysed:

(*a*) in 0·1*N* H_2SO_4 at 100° to the following extents: after 2 min. 81·8%, after 3 min. 94·7%, after 5 min. 99·3%;
(*b*) in a mixture of 0·05*N* HCl and 0·2*N* H_2SO_4 at 100°: after 2 min. 98·5%, after 3 min. 100%.

(2) Galactose-1-phosphate is completely hydrolysed after 2 min. in 0·09*N* H_2SO_4 at 100° [Kosterlitz, 1939*a*].

For the estimation of the sum of the two esters, hydrolysis in a mixture of 0·05*N* HCl and 0·2*N* H_2SO_4 at 100° for 3 min. has been adopted. The HCl is necessary to ensure solution of the Ba salts before the Ba is precipitated with H_2SO_4.

Table 1. *Hydrolysis at 50°*

(1) Hydrolysis of glucose-1-phosphoric acid. Concentration of ester 0·75 m.-molar

Time min.	Glucose formed %	$k \times 10^3$	Inorg. P formed %	$k \times 10^3$
15	15·6	4·91	15·5	4·88
30	29·0	5·01	28·9	5·00
45	40·1	4·92	40·6	5·21
60	49·3	4·83	48·6	4·19
	Average	4·92	—	4·82

Percentage of hydrolysis after 30 min., calculated for $k = 4{\cdot}87 \times 10^{-3}$: *28·55%*.

(2) Hydrolysis of galactose-1-phosphoric acid. Concentration of ester 0·965 m.-molar

Time min.	Galactose formed %	$k \times 10^3$	Inorg. P formed %	$k \times 10^3$
15	51·2	20·8	49·8	20·0
30	78·2	23·3	76·8	22·3
45	89·4	20·9	89·2	22·1
	Average	21·7	—	21·5

Percentage of hydrolysis after 30 min., calculated for $k = 21{\cdot}6 \times 10^{-3}$: *77·5%*.

II. *At 50°.*

The acid used consists of a mixture of 0·2*N* H_2SO_4 and 0·05*N* HCl (Table 1).

After 30 min. the ratio hydrolysed galactose-1-phosphoric acid/hydrolysed glucose-1-phosphoric acid is 2·72/1. If the values for P and for reducing power for the total hydrolysis (3 min. at 100°) and for the partial hydrolysis (30 min. at 50°) are known, the quantities of the two esters can be calculated (Table 2).

Table 2. *Ratios of hydrolysis percentages at 50° and 100° of mixture of glucose-1-phosphate and galactose-1-phosphate*

Mixture of: Glucose-1-phosphate %	Mixture of: Galactose-1-phosphate %	Ester hyd. at 50° / Ester hyd. at 100°	50° value / 100° value calculated from uncorrected P values	50° value / 100° value calculated from reducing power expressed as 'glucose'
100	0	0·286	0·286	0·286
80	20	0·384	0·378	0·364
60	40	0·482	0·472	0·451
40	60	0·579	0·568	0·547
20	80	0·677	0·665	0·654
0	100	0·775	0·765	0·775

Note. The values in column 4 are necessitated by the fact that 4·5% of the galactose-1-phosphate present is hydrolysed during the process of analysis (see note to Table 3), and the values in column 5 by the fact that galactose has only 76·3% of the reducing power of glucose.

Removal of glycogen. A small proportion of the glycogen comes down with the precipitate due to the addition of mercuric acetate but most is precipitated along with the HgS after passing H_2S through the filtrate. Up to 2% liver glycogen is removed in this way. HgS prepared from $HgCl_2$ and H_2S has been used by Flock, Bollman & Mann [1936] for the removal of glycogen.

Effect of mercuric acetate. Mercuric acetate, when added to the neutralized $CCl_3.COOH$ extract, precipitates, amongst other compounds, phosphoric esters containing nitrogenous bases, and consequently the removal of the Hg-precipitate has the following effects: (1) It decreases the 'initial reduction' of purified phosphates by about 75%, e.g. from 61·3 mg./100 g. liver to 14·7 mg. (2) It decreases the labile P (3 min. 100°) considerably, from 0·55 mg. % P (6 exps.) to 0·09 mg. % (10 exps.). (3) It has no influence on the increase in reduction after 3 min. hydrolysis at 100°.

Mercuric acetate also precipitates bile pigments so that a colourless filtrate is obtained.

Removal of inorganic phosphate. This can be done either (1) by precipitating it at *p*H 9·5* as $BaHPO_4$

* *p*H 8·4 is frequently used for the separation as Ba salt of inorganic P from organic phosphates; we found this to be insufficiently alkaline, and therefore used thymolphthalein as indicator which just turns blue at *p*H 9·5.

from the filtrate, after aerating to remove H_2S; removing it by centrifuging and adding 3 vol. ethanol to precipitate the organic phosphates, or (2) as described above, by precipitating the organic and inorganic phosphates together with 3 vol. ethanol, drying the precipitate, and then extracting the organic phosphates. The initial reduction is lower with method (2) than (1), and the increase in reduction in preparations from livers of fasted rats after acid hydrolysis with method (2) is only one-third to one-half of that with method (1). Recovery of added galactose-1-phosphate is the same with the two methods, and since smaller blank values are obtained with method (2) than with method (1) the former has been adopted.

Since $BaHPO_4$ is sparingly soluble in water, small quantities of inorganic P are always found; the average of 13 exps. was 0·55 mg. P/100 g. liver, ranging from 0·35 to 1·03 mg. P. If more water is used for the extraction or the precipitate extracted more than 3 times, the values for inorganic P increase considerably. Extraction with 3 ml. H_2O, repeated twice, is sufficient. Actually the recovery of added galactose-1-phosphate (10·06 mg. P to 30 ml.) from the filtrate after HgS precipitation was 88·5 and 100 % if the precipitate was extracted 5 times with 5 ml. H_2O, and 99 % when successively extracted with 3·5, 2·5 and 1·5 ml. H_2O.

Significance of 'initial reduction'. Although it is likely that the 'initial reduction' is a measure of the amount of equilibrium ester (hexose-6-phosphates) present, there is so far no direct proof of this assumption. Relatively large amounts of glycerol phosphates are present in liver which make it difficult to estimate the P corresponding to the 'initial reduction'. The findings of Cori & Cori [1931–2] suggest that, under the conditions of our experiments, the recovery of hexose-6-phosphates may not be quantitative: the *p*H may be too alkaline and extraction may not dissolve all the 6-phosphates present.

Recovery of galactose-1-phosphate and glucose-1-phosphate added to trichloroacetic acid extracts of liver

Example 1. 2 ml. galactose-1-phosphate solution containing 0·114 mg. P were added to 40 ml. extract (test solution). 1 ml. NaOH was needed for neutralization and 4 ml. 10 % mercuric acetate solution were then added. After centrifuging, a sample of 40 ml. was used. The final volume was 10 ml.; 2 ml. ($\equiv 112\,\mu$g. galactose) were used for the estimation of reduction and 1 ml. ($\equiv 9{\cdot}7\,\mu$g. P) for that of P. Another 40 ml. were treated similarly with the exception that 2 ml. H_2O were added instead of the galactose-1-phosphate solution (blank solution) (Table 3).

Example 2. 1 ml. galactose-1-phosphate solution containing 0·0527 mg. P and 1 ml. glucose-1-phosphate solution containing 0·0524 mg. P were added to 40 ml. extract. This mixture was treated in the same way as the galactose-1-phosphate solution of example 1 (Table 4).

The results of several recovery experiments are summarized in Tables 5 and 6. The standard errors of the means of the recoveries and of the ratios 50° value/100° value were calculated according to the directions given by Burn [1937].

Table 3. *Recovery of galactose-1-phosphate*

	Blank solution 'glucose' or P μg.	Test solution 'glucose' or P μg.	Increases in test after deduction of blank 'glucose' or P μg.	50° value / 100° value	Recovery %
(1) Reduction (in 2 ml. ≡ 112 μg. galactose ≡ 85·5 μg. 'glucose')					
Before hydrol.	28·5	28			
After 3 min. hydrol. at 100°	38·5	105			
Increase	+10	+77	+67		*78·5*
After 30 min. hydrol. at 50°	33	83		*0·755*	
Increase	+4·5	+55	+50·5	(calc. from Table 2: 0·775)	
(2) Phosphorus (1 ml. ≡ 9·7 μg.)					
Before hydrol.	2·86	4·77			
After 3 min. hydrol. at 100°	2·86	11·81			After correction* with $f = 1{\cdot}05$
Increase	0	+7·04	+7·04		*76·2*
After 30 min. hydrol. at 50°	2·43	10·08		*0·755*	
Increase	−0·43†	+5·31	+5·31	(calc. from Table 2: 0·765)	

* This correction is necessitated by the great sensitivity of galactose-1-phosphate to acid. Under the conditions of these experiments, 4·5 % of the ester present is hydrolysed by the analytical procedure alone, therefore the factor for correction is 1·00/0·955 = 1·05. The factor for the 50° value is 0·775/0·73 = 1·06. The amount of glucose-1-phosphate hydrolysed during the analysis is so small as to make a correction unnecessary in the present experiments.

† Occasionally small negative values have been observed after 30 min. hydrolysis at 50°; they have been neglected.

Table 4. *Recovery of galactose-1-phosphate and glucose-1-phosphate*

	Blank solution 'glucose' or P μg.	Test solution 'glucose' or P μg.	Increases in test after deduction of blank 'glucose' or P μg.	50° value / 100° value	Recovery %
(1) Reduction (in 2 ml. ≡ 51·7 μg. glucose + 52·1 μg. galactose ≡ 91·5 μg. 'glucose')					
Before hydrol.	44·5	37·5			
After 3 min. hydrol. at 100°	55·5	121			*79·3*
Increase	+11	+83·5	+72·5		
After 30 min. hydrol. at 50°	53·5	80		*0·462*	
Increase	+9	+42·5	+33·5	(calc. from Table 2: 0·498)	
(2) Phosphorus (1 ml. ≡ 8·95 μg.)					
Before hydrol.	1·99	2·26			
After 3 min. hydrol. at 100°	2·30	8·57			After correction with $f = 1·02$ *68·5*
Increase	+0·31	+6·31	+6·0		
After 30 min. hydrol. at 50°	1·91	5·23		*0·495*	
Increase	0	+2·97	+2·97	(calc. from Table 2: 0·520)	

Table 5. *Recovery of glucose-1-phosphate*

2·65–3·1 mg. ester P/100 g. liver

Amount of ester P added to extract mg./40 ml. (≡4 g. liver)	Glucose liberated in 3 min. at 100° % of calc.	Glucose liberated in 30 min. at 50° calc. = 28·6	50° value / 100° value calc. = 0·286	P liberated in 3 min. at 100° % of calc.	P liberated in 30 min. at 50° calc. = 28·6	50° value / 100° value calc. = 0·286
0·106	85·4	23·5	0·276	85·2	27·5	0·323
0·111	88·2	26·6	0·302	75·5	18·6	0·246
0·111	91·5	25·0	0·273	87·2	26·9	0·309
0·124	83·3	22·6	0·272	81·0	21·4	0·265
Mean	87·1 ± 1·78	24·4 ± 0·88	0·280 ± 0·0116	82·2 ± 2·59	23·6 ± 2·16	0·287 ± 0·0278
Coefficient of variation (%)	2·04	3·61	4·15	3·15	9·15	9·68

Table 6. *Recovery of galactose-1-phosphate*

(*a*) 2·68–2·85 mg. ester P/100 g. liver

Amount of ester P added to extract mg./ml.	Galactose liberated in 3 min. at 100° % of calc.	Galactose liberated in 30 min. at 50° calc. = 77·5	50° value / 100° value calc. = 0·775	P liberated in 3 min. at 100°, corrected % of calc.	P liberated in 30 min. at 50°, corrected calc. = 77·5	50° value / 100° value corrected calc. = 0·775
0·12/40 (≡4 g. liver)	76·5	56·6	0·74	73·8	56·9	0·77
0·12/40	84·5	60·4	0·715	83·6	61·0	0·73
0·114/40	78·5	58·7	0·75	76·2	58·3	0·765
Mean	79·8 ± 2·40	58·6 ± 1·10	0·735 ± 0·0261	77·9 ± 2·95	58·7 ± 1·20	0·755 ± 0·0325
Coefficient of variation (%)	3·01	1·88	3·55	3·79	2·04	4·30

(*b*) 0·60–1·5 mg. ester P/100 g. liver

0·06/40 (≡4 g. liver)	72·5	52·0	0·715	69·8	60·0	0·86
0·06/40	72·5	40·6	0·56	91·6	70·9	0·775
0·057/40	86·0	64·3	0·75	80·9	54·4	0·675
0·0306/50 (≡5 g. liver)	95·0	65·5	0·69	68·7	54·1	0·785
0·0301/50	70·5	70·5	1·00	75·1	58·8	0·785
0·0422/50 containing 0·2% glycogen (≡ 2% liver glycogen)	69·0	58·3	0·845	72·9	49·1	0·675
Mean	77·6 ± 4·28	58·5 ± 4·43	0·754 ± 0·0705	76·5 ± 3·50	57·9 ± 3·05	0·757 ± 0·0528
Coefficient of variation (%)	5·51	7·57	9·36	4·58	5·27	6·98

In the recovery experiments the esters were added to liver extracts in proportions similar to those found in determinations of these esters in livers of animals assimilating glucose or galactose. Approximately 80 % of the added esters were recovered; there was no significant difference between the recoveries of hexose and phosphorus. The ratios 50° value/100° value were sufficiently close to the calculated values. As was to be expected, the standard errors of the means were relatively high in the experiments in which only 0·6–1·5 mg. ester P/100 g. liver was added. These facts have to be taken into consideration when experiments are designed. The limiting factors obviously are the methods used for the estimation of reducing power and of phosphorus. These difficulties are best overcome by increasing the quantities of liver used for the estimation but, unfortunately, in the rat at least, this cannot easily be done, particularly when other substances, as, for example, glycogen and hexose, have to be determined simultaneously.

SUMMARY

1. Glucose-1-phosphate and galactose-1-phosphate are determined in trichloroacetic acid extracts of rats' livers. After removal of interfering reducing substances, i.e. nitrogenous phosphoric esters, bile pigments, and glycogen, by means of mercuric acetate and HgS, the organic phosphates are separated from inorganic P as water-soluble barium salts.

2. The reducing power of the organic phosphate fraction gives an approximate measure of the amount of hexose-6-phosphates present. The quantity of glucose-1-phosphate and galactose-1-phosphate taken together is determined by the increase in reducing power and the amount of inorganic P liberated after hydrolysis in a mixture of 0·05*N* HCl and 0·2*N* H_2SO_4 at 100° for 3 min. Incubation with 0·25*N* acid at 50° for 30 min. hydrolyses 28·6 % of glucose-1-phosphate and 77·5 % of galactose-1-phosphate. In a mixture of the two esters, the ratio 50° value/100° value is determined by the relative concentrations of the esters.

3. In experiments with addition of known quantities of ester to $CCl_3.COOH$ extracts of liver, 84·5 % of added glucose-1-phosphate (2·65–3·1 mg. P/100 g. liver), 79 % of added galactose-1-phosphate (2·68–2·85 mg. P/100 g. liver), and 77 % of added galactose-1-phosphate (0·6–1·5 mg. P/100 g. liver) were recovered. The ratios 50° value/100° value did not show any significant deviation from the expected values. The significance of the standard errors of the means of the results for the recovery of added material is discussed.

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An Improved Method for the Fractionation of Protein Mixtures by Electrophoresis

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The application of the electrophoresis technique to the problem of analysing protein mixtures was greatly refined by Tiselius [1937 *a*, *b*], who used the method particularly for the analysis of serum proteins. Since then it has been used by a variety of workers, Kekwick & Record [1940], for instance, having applied it to the isolation of the biologically active globulins from immune horse serum. Although the method is of great value, only relatively small quantities of proteins can be separated by it and at a high cost. Moreover, attempts to increase the yields have met with considerable difficulties [Philpot, 1940].

In their purification of a variety of colloids by electrodialysis between two semi-permeable membranes, Pauli, Stamberger & Schmidt [1937] ob-

served the formation, in the fluid within the membrane cell, of superposed layers of different colloids [cf. Blank & Valko, 1928]. This is caused by the colloid particles' migrating to the membrane and concentrating at its surface. If the current density is not too great the colloidal particles are then more under the influence of gravity than of the electric field, and they move along the vertical face of the membrane, downwards if their specific gravity is greater than that of the dispersion medium, or upwards if it be less. These findings were applied to the concentration of rubber latices by Pauli *et al.* [1937].

It seemed that it might be possible to apply the method to the separation of protein mixtures. Thus, if the electrodialysis of a mixture were carried out in a buffer solution at the *p*H of the isoelectric point (I.E.P.) of one particular protein, under suitable conditions of current density and of time, the other proteins would move to one or other of the membranes and there concentrate in the lower layers, thus leaving the required protein in solution in the upper layers. This could then be removed from the cell by appropriate means. By repeating the process at different *p*H's it should then be possible, by successive treatments, to resolve the mixture into a series of electrically homogeneous constituents. This process we designate 'electrodecantation'.

METHODS

Fig. 1 shows the elevation, through the middle of the glass parts, of the apparatus. It consists of two outer glass cells *A*, the circular open ends of which are covered by cellophane membranes *C*; the closed cells so formed are separated from

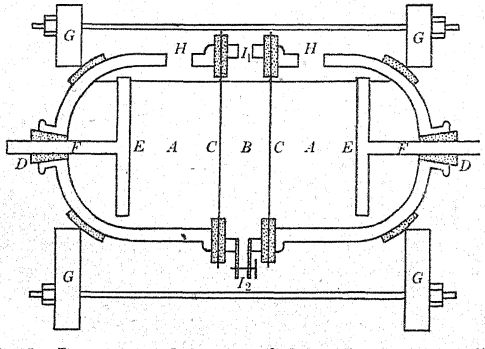

Fig. 1. Longitudinal section of electrodecantation cell.

each other by a short cylinder of glass, so that when clamped together, the middle cell *B* is formed. The inner radius of the ends of the cells *A*, which carry the membranes, is 11 cm., and the distance from the membranes to the inner surface of the rubber bungs *D*, through which the electrodes are inserted, is 9 cm.; the width of the glass cylinder separating the outer cells from each other is 1·8 cm. Each electrode, *E*, consists of a circular carbon disk of diameter 9 cm. and is connected to a 12 V. battery by a carbon rod *F*, passing through the rubber bungs *D*; a voltmeter and an ammeter are included in the circuit. For clamping the apparatus together two vertical boards of hard wood, 18 × 22 cm., *G*, are used, the shorter edge being used as the base; these boards also serve as a stand for the apparatus. Each has a circular hole of diameter 7 cm., the lower edge of which is 11 cm. from the base, so that the neck of the cells *A* may be inserted. Four bolts, 15 cm. long, passing through the two wooden boards, are used to clamp the apparatus, by pressure on the outer surface of the cells *A*, heavy rubber washers being inserted between the boards and the glass surface of the cells *A* to prevent fracture; thin rubber washers of width 1 cm. are placed between cells *A* and *B*.

Holes in the cells *A*, through which the buffer solutions may be introduced or withdrawn, are provided at *H*, while holes for the introduction and removal of the protein solutions are provided in the middle cell *B* at I_1 and I_2.

Investigation of method

For test purposes a mixture of ovalbumin and haemoglobin was used. The ovalbumin was prepared by dissolving purified egg albumin (B.D.H., Ltd.) in distilled water, filtering and adding to the filtrate an equal vol. of sat. $(NH_4)_2SO_4$; after standing overnight at 0°, the mixture was filtered and dialysed against distilled water. When free from $(NH_4)_2SO_4$ the albumin solution was dialysed against a suitable buffer of the *p*H required in each particular experiment.

The haemoglobin was prepared by haemolysis of human red cells which had been washed well with isotonic saline. The concentrated crude solution was precipitated with sat. $(NH_4)_2SO_4$, dialysed first against distilled water, and then against buffer solutions of the required *p*H, as described for the albumin.

The buffer solutions used throughout consisted of suitable mixtures of 0·2*M* NaH_2PO_4 and 0·1*M* citric acid. The electrolysis was carried out at 0° at a potential of 1 V./cm. with a current of 0·8 amp.; the duration of each run was 6 hr. It was found by a series of experiments with haemoglobin solutions that at *p*H 5, after 6 hr. of electrodecantation, the upper one-third of the solution in the middle cell was free from haemoglobin; a similar result was obtained with egg albumin solutions at *p*H 7.

RESULTS

After the preliminary experiments, the separation of haemoglobin from egg albumin was investigated. Solutions of haemoglobin and egg albumin were

prepared as described, and these were finally dialysed against several changes of buffer at the approximate I.E.P. of haemoglobin, i.e. *p*H 6·8. Total N was determined on each solution, and the protein concentration was taken as 6·25 × N. Suitable volumes of each solution were then mixed together to yield a solution containing 1·1 % haemoglobin and 0·9 % albumin. The solution was submitted to electrodecantation at *p*H 6·8, under the conditions already described. After 6 hr. the middle cell (capacity 240 ml.) was emptied by running out twelve 20 ml. portions very slowly from the tap at I_2. The total protein content of all the fractions so obtained was determined by Kjeldahl nitrogen estimation, the haemoglobin in each fraction being estimated colorimetrically against a standard prepared from the original haemoglobin solution. The concentration of albumin and haemoglobin in each fraction was then calculated. In Table 1 the first fraction (from the bottom of the cell) is numbered 12, while 1 represents the last 20 ml. from the top of the cell.

Table 1 shows that fractions 1–4 were completely free of albumin, and that a progressive increase in the albumin concentration occurred as the bottom of the cell was approached, the last fraction containing 2·5 g./100 ml. of this protein. In 6 hr. it is possible thus to free one-third of the cell contents from albumin, and so to obtain one-third of the haemoglobin as an electrochemically homogeneous fraction. It had been anticipated that the haemoglobin concentration would remain constant in all 12 samples. Actually, as Table 1 shows, the fractions 1–6 retained the original concentration of 1·1 g./100 ml., fraction 7 was lower, i.e. 1·0 g./100 ml., while the remaining 5 fractions contained 0·8 g./100 ml. giving an average concentration of 0·96 g./100 ml., an apparent loss of about 13 % of the original haemoglobin. This apparent loss was due to precipitation. When the haemoglobin content of each fraction was estimated on the cleared solution the precipitated haemoglobin remained unestimated; it was included, however, in the total N content, which was determined on a well-shaken sample. Thus in Table 1 the figures for albumin in the lower fractions are slightly higher than they should be.

Table 1. *Concentration of albumin and haemoglobin at different levels in the cell*

No. of sample	Albumin g./100 ml.	Haemoglobin g./100 ml.
1 (top of cell)	0·0	1·1
2	0·0	1·1
3	0·0	1·1
4	0·0	1·1
5	0·2	1·1
6	0·4	1·1
7	1·4	1·0
8	1·7	0·8
9	2·1	0·8
10	2·4	0·8
11	2·4	0·8
12 (bottom of cell)	2·5	0·8

The reason for the precipitation of a small fraction of the haemoglobin is not clear. The *p*H at which the electrodecantation was carried out was deliberately chosen to be slightly on the alkaline side of the I.E.P., and doubtless there was a slight migration of the haemoglobin under these conditions with a subsequent precipitation of a small part of it.

DISCUSSION

Although the methodi s still in the experimental stage, these results indicate that it should be applicable to many biological problems where the isolation of homogeneous protein fractions is involved. Thus, at the present time, it is being used for the separation of the biologically active globulins of immune horse serum. The apparatus is simple, and considerable quantities of material can be obtained rapidly at a low cost. There is, moreover, no theoretical reason why the apparatus should not be greatly enlarged, with a corresponding increase in output. The apparatus may be improved by the introduction of modifications to allow a continuous change of buffer solution in the outer cells, and by the provision of suitable taps, placed at different levels in the central compartment, to facilitate the removal of successive layers of the cell contents at the close of an experiment.

The optimum conditions of *p*H, time, and current density, for separation of different protein mixtures requires individual investigation.

SUMMARY

1. A preliminary description of a new method for the separation of protein mixtures is described.

2. The method, which depends on electrophoresis, possesses an advantage over the method of Tiselius in that it makes possible the preparation of considerable quantities of electrically homogeneous protein fractions.

3. The application of the method to the separation of mixtures of albumin and haemoglobin is described.

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The Reaction of Heparin with Proteins and Complex Bases

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Owing to its strongly acid nature, heparin forms stable salts with many proteins, examples of which have been reported by a number of workers, e.g. Reiner & Kopp [1928], Fischer [1931], Chargaff & Olsen [1937], and Scott & Fisher [1942]. The general nature of the reaction was first indicated by Fischer [1935] and Fischer & Astrup [1935] who studied its kinetics. While other examples of the reaction are now being reported, many authors appear to have overlooked these important contributions by Fischer, and in particular his finding that the reaction is a simple salt formation in which reversible dissociation of the salt occurs according to the mass law [Fischer & Astrup, 1935]. This latter point means that data may be analysed more thoroughly than hitherto, and that confusing results have frequently a simple physico-chemical explanation. During studies on heparin, the author has frequently confirmed Fischer's original observations in experiments carried out as controls for other studies. These findings, and the results of other experiments, are reported in this communication.

Three principles are available for the demonstration of the reaction of heparin with proteins. These are: (1) direct observation of the formation of the compound, e.g. precipitation of the gelatin-heparin compound when the two solutions are mixed; (2) the alteration in the properties of the protein, e.g. the shift in the isoelectric point (I.E.P.) of casein; the decrease in precipitability of serum globulin by alcohol, heat, and other precipitating agents [Fischer, 1935]; loss of activity by fumarase [Fischer & Herrmann, 1937]; (3) alteration in the properties of heparin, e.g. loss of dialysing power with egg albumin [Fischer, 1935] and loss of anticoagulant activity with protamines [e.g. Fischer, 1935; Chargaff & Olsen, 1937]. In our experience, it is necessary to choose a suitable method for each protein.

Failure to observe compound formation does not necessarily indicate that it has not occurred. Thus when the heparin-protein compound is soluble under the conditions of the experiment (e.g. protamine in the presence of plasma) precipitation of the compound cannot be observed. The reaction can, however, still be detected by suitable indirect means (in this case by the loss of anticoagulant activity of the heparin).

Fischer indicated that a stoichiometric relationship existed between heparin and thromboplastin (and that presumably this extended to other proteins). Fischer & Astrup [1935] investigated this stoichiometric relationship more fully and found that the reaction could be represented as

$$\text{Protein} + \text{heparin} = \text{protein-heparin}.$$

$$\text{From the mass law, } \frac{C_{\text{protein}} \times C_{\text{heparin}}}{C_{\text{protein-heparin}}} = K.$$

Then, if a be the concentration of protein added, c the concentration of free protein, and b the concentration of added heparin, $a-c$ is the concentration of the product formed by the reaction, $b-(a-c)$ is the concentration of free heparin, and

$$K = \frac{c\{b-(a-c)\}}{a-c}. \qquad (1)$$

Fischer & Astrup [1935] obtained satisfactory agreement with this equation for the mutual interaction of thromboplastin and heparin on hen plasma. The author has also found this to give good agreement for the reaction of heparin with casein, protamine, and toluidine blue, a non-protein basic substance. On the basis of these and other experiments, it seems likely that it is true of the reaction with all proteins, sufficient reversible dissociation taking place to have a significant effect on the kinetics of the reaction. The data of other authors show no disagreement with this view and tend to corroborate the general nature of the reaction. Actually, the earliest studies on heparin indicated that the biological action of heparin depended on such a reaction, since they showed that peptone

plasma could be clotted by simple dilution, i.e. by increasing the dissociation of the heparin-protein compound its anticoagulant effect could be annulled [Morawitz, 1905].

METHODS

Heparin was supplied by the Connaught Laboratories, University of Toronto, and had been crystallized as the barium salt. The sodium salt has also been used. Heparin was assayed either by the Howell cat method [Jaques & Charles, 1941], or by means of its metachromatic reaction with toluidine blue, when the Lovibond tintometer was used. The temperature for all experiments was 25°. I am indebted to Dr A. M. Wynne of the Department of Biochemistry for the use of an Evelyn photoelectric colorimeter.

Reaction of heparin with casein. This reaction is easily demonstrated, because although casein has an I.E.P. at *p*H 5·0, that of the casein-heparin compound is at *p*H 3·6. The compound is soluble at *p*H 5·0, and Fischer & Schmitz [1932] used this fact in assaying heparin. The I.E.P. of the casein was in the region of *p*H 4·6–4·9 (found value *p*H 4·8), whereas that of the casein-heparin was in the region of *p*H 3·5–4·3 (Table 1). The shift is as marked as that with eosin [Jordan-Lloyd & Shore, 1938]. The reaction was then studied at *p*H 5·0 by adding 1 ml. casein solution (50 mg./100 ml. *N*/10 Na acetate) to 9 ml. of *N*/180 acetic acid containing varying amounts of heparin and measuring the turbidity in an Evelyn colorimeter (Table 2). The turbidity values were converted to mg. of free casein by the experimentally determined equation $C = 3{\cdot}96T + 0{\cdot}0261$. Owing to the small portion of the scale used for the determination of turbidity, a considerable spread in the experimental values was found. The calculated values of K are given in Table 2, and show fairly good agreement. For convenience these values were calculated in terms of units of heparin and mg. of casein.

Reaction of heparin with toluidine blue. This important reaction was reported by Jorpes [1939] for use as a histological test for heparin. It consists in measuring the red colour of the compound. The reaction was studied with the aid of the Lovibond tintometer. A sample of Groebler's toluidine blue was used, 1 ml. being added to 4 ml. of heparin solution in saline containing 0·25 % Ca chloride. Table 2 shows that this compound formation is of the same type as that produced with proteins, the results showing good agreement with the mass law.

Table 1. *Effect of heparin on the isoelectric point of casein*

− no precipitation. + degree of precipitation.

*p*H	Casein + Heparin		*p*H	Casein + Heparin	
5·80	±	−	4·34	+ +	+ + +
5·31	+	−	4·14	+ +	+ + +
4·88	+ +	+	3·52	−	+ + +
4·78	+ + +	±	1·90	−	±
4·56	+ +	+ +			

Casein (B.D.H. light white soluble), 1 mg./ml. Heparin, 0·1 mg./ml. Buffer, 0·2 *N* K acetate-acetic acid. *p*H measurements by the Beckmann glass electrode.

Table 2. *The reaction of heparin with casein and toluidine blue*

a (amount of casein added) = 0·5 mg. *p*H = 5·0

Heparin added mg. × 10^{-2} = b	Turbidity	Free casein mg. = c	$K = \frac{c\{b-(a-c)\}}{a-c}$
0·00	0·1206	0·503	—
0·153	0·1065	0·447	0·84
0·459	0·0757	0·326	0·53
0·765	0·0655	0·285	0·73
1·22	0·0518	0·269	0·82
1·53	0·0339	0·160	0·56
1·53	0·0458	0·208	0·88
4·59	0·0155	0·270	0·88
7·65	0·0044	0·0435	0·69
15·3	0·0000	0·000	—
		Average	0·72

a (amount of toluidine blue added) = 5 mg./100 ml. *p*H = 5·0

Heparin mg. × 10^{-2}	Tintometer red reading	Combined dye mg./100 ml. = $a-c$	K
2·0	0·5	1·5	1·00
4·0	3·5	2·6	1·54
5·0	4·8	3·1	1·81
6·0	6·0	3·5	1·65
7·0	6·5	3·7	1·75
8·0	6·5	3·7	1·64
		Average	1·56

Reaction of heparin with gelatin. While the reaction with casein is readily detected by the markedly different I.E.P.'s of the protein and of its compound with heparin, the reaction with gelatin, and also that with protamine, can be studied over a wider *p*H range, since the compounds formed between these proteins and heparin were found to be relatively insoluble over the range *p*H 2·5–10·5. Increasing amounts of gelatin were added to 2·4 mg. of heparin. The mixtures were centrifuged and the N content of the precipitates determined by the micro-Kjeldahl method. The heparin present in the supernatant was determined colorimetrically, that in the precipitate being calculated by difference. It can be seen (Table 3) that increasing the concentration of gelatin first increases, and then decreases, the amount of precipitate, i.e. the precipitate is soluble in the presence of an excess of gelatin. The composition of the precipitate, however, remains unchanged. It should be pointed out that not all samples of gelatin form an insoluble heparin compound. For these experiments the gelatin used was

an ordinary commercial gelatin, but a sample of Eastman Kodak technical gelatin, and also a sample of carefully prepared fish collagen (courtesy of Prof. N. B. Taylor) failed to give any precipitate with heparin over a wide range of concentration and *p*H.

Reaction of heparin with protamine. Chargaff & Olsen [1937] observed that when heparin and protamine are mixed an insoluble complex is formed, and showed by direct analysis of the complex that a simple salt formation had occurred. We mixed the protamine, salmine (Connaught Laboratories) and heparin in a total volume of 5 ml. containing 0·5 ml. of 10 % $CaCl_2$, the amount of precipitate formed being estimated by determining its N content by the micro-Kjeldahl procedure (Table 3). The values obtained for the dissociation constant showed that with this reaction also, partial dissociation of the compound occurs.

Table 3. *Reaction of heparin with gelatin and protamine (salmine)*

1 ml. of heparin (2·4 mg.) added to 3 ml. of gelatin solution. *p*H = 3·9.

Gelatin N added mg.	N in ppt. mg.	Heparin in ppt. mg.	N/heparin in ppt.
0·66	0·050	0·26	—
1·31	0·714	1·53	0·47
2·62	1·41	2·06	0·68
4·58	0·798	1·85	0·43
6·55	0·606	0·97	0·62
13·1	0·122	0·26	0·48
26·2		No precipitate	

Heparin added to the protamine, salmine (Connaught Laboratories). Total volume = 5 ml. *p*H = 2·5. $CaCl_2$ concentration = 1 %.

Heparin mg.	Protamine mg. N	N in ppt. mg.	*K*
0·1	0·347	0·050	0·297
0·2	0·347	0·087	0·338
0·5	0·347	0·150	0·398
1·0	0·347	0·244	0·319
1·0	0·173	0·174	—
1·0	0·069	0·066	—
1·0	0·034	0·030	—
		Average	0·338

Inactivation of heparin by proteins. As Fischer [1935] first indicated, certain proteins possess the power, not only of combining with heparin, but also of inactivating this anticoagulant. Chargaff & Olsen [1937] suggested the use of this reaction to inactivate heparin both *in vitro* and *in vivo*. The proteins possessing this property in the highest degree are the protamines, salmine and clupein. The protamine was thought to inactivate heparin by precipitating the latter, as in the above studies. If, however, protamine and heparin are mixed in the presence of plasma and in the concentrations normally used, precipitation does not occur, although inactivation has taken place. That this is so can be seen by testing for the anticoagulant activity of heparin. This shows that protamine has combined with heparin to form an inactive compound.

The reaction can be further studied by adding increasing amounts of protamine to heparinized blood and determining the clotting time. As reported by Jaques, Charles & Best [1938] the clotting time decreases until it reaches the normal value found in the absence of heparin, and then increases again. In the presence of excess heparin or protamine the clotting time is prolonged and it is only at the equivalence point that the normal clotting time obtains. At the equivalence point the ratio of heparin to protamine was constant. This is true of any one protamine preparation, but more recent work has indicated that it is different for different preparations. Such a protamine titration is reported in Table 4. The experiment was carried out as previously described [Jaques *et al.* 1938], using the protamine, salmine, and adding 1 ml. of freshly drawn dog's blood to various mixtures of heparin and protamine. Also reported are the clotting times with heparin, and protamine alone. It can be seen that 0·5 mg. of heparin required 1·62 mg. of protamine to restore the clotting time to normal, i.e. 3·2 mg. protamine/mg. heparin. The same equivalence value was obtained with 0·1 mg. of heparin. It was shown that the reaction between protamine and heparin could be expressed by equation (1). The data indicate that in the presence of serum also dissociation of the protamine-heparin compound occurs. Thus the equivalence value for 0·5 mg. of heparin is 1·62 mg. protamine, whence 1·37 mg. protamine would combine with 0·42 mg. heparin. Thus there would be 0·08 mg. free heparin which would give a clotting time of over 24 hr. (0·008 mg. heparin gave a clotting time of 2 hr.). However, the clotting time with 1·37 mg. protamine was only 9 min. (corresponding to 0·002 mg.). The reason for this follows from the previous discussion, i.e. the large amount of protamine at the equivalence point is not that required to combine with heparin, but that required to suppress dissociation of the protamine-heparin compound. The values of the mass law dissociation constant calculated for the clotting times obtained with 0·5 mg. heparin were 0·39, 0·33, $0{\cdot}44 \times 10^{-2}$ (average $= 0{\cdot}39 \times 10^{-2}$). The average value of *K* for a sample of thymus histone, calculated from the data reported by Jaques *et al.* [1938] was $0{\cdot}76 \times 10^{-2}$. Clearly, this dissociation is responsible for the sharpness of the titration of heparin with protamine. Protamine is only a weak anticoagulant, but such a large excess of the protein is required to suppress completely the dissociation of the compound that the concentration of protamine need be increased only slightly for it to affect the clotting time.

Fischer [1935] reported that a number of proteins possess the power to inactivate heparin. These were chiefly basic proteins, such as the protamines, histones and haemoglobin. Inactivation also occurs with other proteins, such as the liver proteins. In this case it is necessary to incubate the protein and heparin at an acid *p*H. Denaturation of the liver protein by heating results in the inactivation of more heparin. We have confirmed Fischer's results, using a saline extract of dog liver. Such experiments require careful controls, since the acid protein solution may lower the *p*H of the test blood thus making it less sensitive to heparin [Fischer, 1935], and suggesting that inactivation of the latter has occurred. Thus failure of the protein to annul the anticoagulant activity of heparin does not indicate that compound formation at the *p*H of the blood does not occur. For example, a gelatin-heparin mixture, in which almost all the heparin was found to be combined with the gelatin when the precipitate was removed and analysed, still showed full biological activity.

Table 4. *The effect of heparin and protamine on clotting time*

Heparin mg.	Clotting time (min.)	Protamine mg.	Clotting time (min.)
—	3	0·017	3
—	2	0·025	4
0·002	9	0·05	5
0·003	22	0·10	7
0·004	27	0·25	5
0·006	54	0·25	8
0·008	113	1·00	70

Protamine (mg.) added to 0·1 mg. heparin	Clotting time (min.)	Protamine (mg.) added to 0·5 mg. heparin	Clotting time
0·25	5	1·00	2 hr.
0·30	4	1·25	15 min.
0·32	2	1·37	9 min.
0·35	3	1·50	11 min.
0·40	6	1·62	2 min.
0·75	10	2·00	1 hr.

Factors influencing the dissociation constant of the heparin-protein compound. The dissociation of these compounds is markedly affected by the *p*H and the presence of other anions and cations (Table 5 and Fig. 1). These results have been obtained chiefly with toluidine blue, since it is relatively simple to obtain quantitative data with this base. Results obtained with a number of different proteins, however, indicate that the same conclusions apply to them. A change in *p*H from 4·1 to 6·2 (Table 5) resulted in a tenfold increase in the dissociation constant. Hence, as with salts of many organic bases, the corresponding heparin compound may often be completely hydrolysed by warming with dilute alkali, the free base being precipitated. The presence of various cations and anions also results in increased dissociation of these compounds since they compete for the heparin and the base respectively. The presence of 0·5 % $CaCl_2$ (Table 5) increased the dissociation constant fivefold. Since the equilibrium depends on the relative dissociation constants of the protein (or dye)-heparin compound and of the salts of protein and heparin with the inorganic anion and cation respectively, further increase in the concentration of the inorganic salt results in the removal of a large proportion of the base from the heparin (Fig. 1). Of the three salts shown, NaCl is as effective as $CaCl_2$, suggesting that the effect is not due to the cation alone. The presence of proteins such as protamine or those of horse plasma similarly decreases the amount of heparin bound to the dye. Sufficient citrated horse plasma was included in one series to give a final concentration of plasma of 60 % by vol. and in another series protamine (2 mg./100 ml.) was added. In each case less heparin combined with the dye. The protein is here acting as a cation, and it is interesting that even the weak plasma protein can act in this manner if sufficiently concentrated. The presence of a strong acid such as $CCl_3.COOH$ also results in the dissociation of the compound (Fig. 1). 5 % $CCl_3.COOH$ completely inhibited the formation of the heparin-dye compound, even when a very large excess of heparin was present. 1 % $CCl_3.COOH$ decreased the amount of heparin bound to dye by 50 % when 0·05 mg. heparin was present.

Impurities in crude heparin preparations may exhibit the type of competitive inhibition shown in Fig. 1 to a marked degree. Thus, during assays by the dye reaction, it was found that the preparation showed no activity in concentrated solution. On diluting the preparation, however, heparin activity could be demonstrated. The effect of NaCl on the antithrombin activity of heparin, similar to that shown in Fig. 1 for its reaction with toluidine blue, has been reported previously [Jaques & Mustard, 1940]. In contrast to these results, Fischer reported that the presence of lipoid increased the binding power of proteins for heparin.

Table 5. *Effect of changes of p*H *and of the presence of calcium chloride on the dissociation constant of the heparin-base compound*

Base	*p*H	*K*
Thymus histone	2·5	0·038
	4·1	0·043
	6·2	0·288
	8·2	1·68
	$CaCl_2$ (%)	
Toluidine blue	0·0	0·60
	0·1	1·16
	0·2	1·56
	0·5	3·06

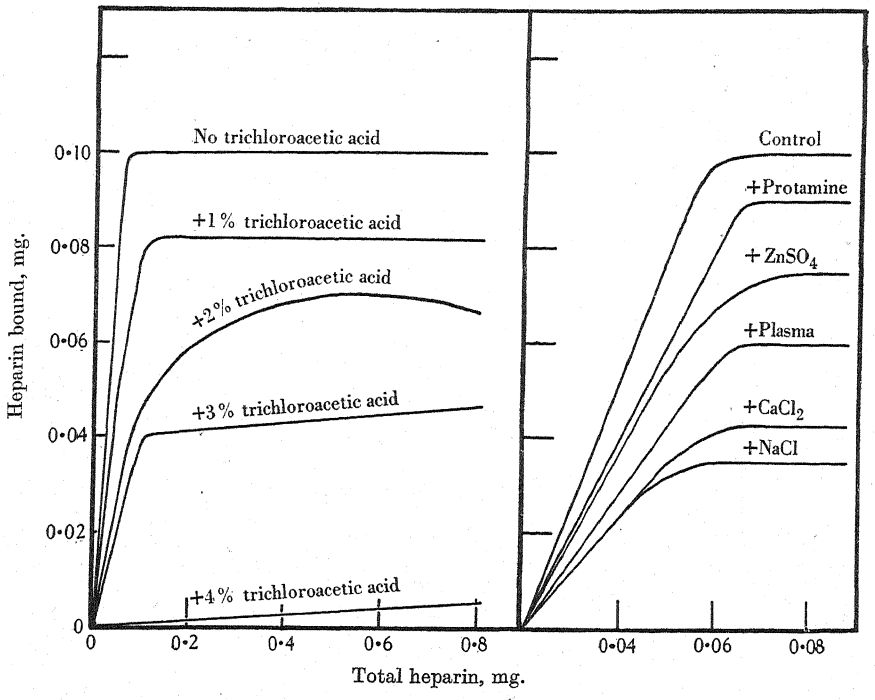

Fig. 1. Effect of various factors on the reaction of heparin with toluidine blue. *p*H 9·8 (borate buffer). Final concentration of reagents, dye, 5 mg./100 ml.; $ZnSO_4$, $CaCl_2$, NaCl, 5%; plasma, 60% (=4% protein); protamine, 2 mg./100 ml. $CCl_3.COOH$ neutralized with NaOH.

DISCUSSION

The proteins here used were selected to illustrate certain extensions of Fischer's theory. As Fischer [1935] established, the reaction is a simple salt formation between the acid heparin and the basic protein. As such, it obeys the mass law so long as the product remains in solution. When, however, precipitation of the product occurs, there may be considerable deviations from the mass law. This is to be expected since these substances possess a high electrostatic charge and the calculations are necessarily based on concentrations and not on activities. Since heparin possesses two types of acid groups and there are differences between the various amino-groups of a protein, there is also the possibility that combination of heparin and protein may occur in more than one proportion, i.e. a series of compounds of the general formula P_xH_y (P=protein, H=heparin) may be obtained. Heidelberger & Kendal have discussed this type of reaction with respect to the precipitin reaction. As reported above, the reaction between heparin and certain samples of gelatin shows an equivalence point and an inhibition zone, as in the antigen-antibody reaction. Since, however, the composition of the precipitate does not change under these conditions, the inhibition phenomenon is probably best explained in terms of the electrostatic theory of electrolytes than by complex compound formation.

Fischer stated that combination between heparin and protein occurs only near the I.E.P., and on its acid side. With histone and gelatin however, this appears not to be the case. Although a shift in *p*H towards the alkaline side increases the dissociation of the compound, it is possible for heparin to combine with proteins far on the alkaline side of their I.E.P., e.g. gelatin precipitates with heparin even at *p*H 10. Hence, the greater part of the heparin in plasma must be bound to protein and not in the free state as suggested by Fischer. Further, it is possible by using a strong acid such as $CCl_3.COOH$ to bring about dissociation of the heparin-protein compound even on the acid side of the I.E.P.

Two values are important in a quantitative study of the reaction between heparin and a given protein. One is the amount of protein which combines with 1 mg. of heparin, the other is the dissociation constant of the compound so formed. The former is best determined by preparing and analysing the compound, although Fischer & Astrup [1935] have suggested a possible graphical method. Since the molecular weight of heparin is not known, it is impossible to calculate the true dissociation con-

stant for even the simplest of these compounds. From equation (1), a function related to the dissociation constant can be obtained, but the concentration of the base must be expressed in terms of the equivalent amount of heparin, if it is desired to compare these values for different proteins. This has not been done in the case of the values so far reported, the constant having been calculated directly from the experimentally determined values in each instance.

The amount of protein which neutralizes the anticoagulant activity of 1 mg. of heparin is also of practical interest. This depends both on the amount of protein required to combine with the heparin and on the dissociation constant of the compound. Thus 4·1 mg. of toluidine blue combined with 1 mg. of heparin, but 7·1 mg. of the dye was required to neutralize its anticoagulant activity. The reaction represents a kinetic equilibrium, and if a second protein (or other cation) be added to a solution of heparin+protein the heparin will distribute itself between the two proteins, according to their relative concentrations and to the dissociation constants of the corresponding heparin compounds. Because the dissociation constant of the gelatin-heparin compound is greater than that of the compounds formed by heparin and the proteins of the clotting system in blood, if it is added to blood, practically all the heparin is transferred to the clotting system and its anticoagulant effect becomes apparent. If it is replaced by the protamine-heparin compound, however, no anticoagulant effect is observed, because the dissociation constant of the latter is smaller than those of the compounds formed by heparin with blood proteins.

Our general conclusions should be applicable to the reaction of heparin with any protein with which it forms such compounds, e.g. silk peptone, gelatin, clupein, salmine, histones prepared from thymus and blood, haemoglobin, egg albumin, casein, serum proteins, liver proteins, coagulant tissue extracts and thromboplastic protein, fumarase and trypsin. Reaction has also been shown to occur with a number of organic bases, namely, benzidine, quinine, brucine, piperidine, and the basic dyes, thionin and toluidine blue. When the protein involved exhibits enzymic or other biological activity the heparin will have an inhibitory effect, e.g. it has a slight inhibitory action on complement. Since the heparin salts of proteins are often entirely different from their parent proteins, showing different solubilities, I.E.P.'s, etc. (the criteria usually employed to classify proteins), the question arises as to whether heparin also affects the antigenic properties of such proteins. Kyes & Strauser [1926] reported that heparin inhibited anaphylactic shock in pigeons sensitized to sheep serum, and this was decisively confirmed by Van de Carr & Williams [1928], who used the sensitized guinea-pig uterine strip (Dale). A number of other workers, including the author, have failed to confirm these findings. It is clear, however, from the previous discussion on the reaction between heparin and proteins, that such inhibitory action will depend to a high degree on the relative concentrations of heparin and protein, on the nature of the protein (including the degree of sensitization) and on the other proteins present. Hence it may be difficult to reproduce the necessary conditions.

Fischer & Astrup established that this type of reaction occurred between thromboplastin and heparin, the overall effect of the two on the clotting system agreeing with the values calculated from the mass law. Chargaff, Ziff & Cohen [1940] studied the same reaction isolated from the clotting system and found that in aqueous solution thromboplastin could be made by shaking to dissociate into cephalin and protein. They also found that when heparin was added to thromboplastin it displaced the cephalin in it. Our explanation would be that dissociation of cephalin and protein had occurred, followed by combination of heparin and protein. As the second compound would have a lower dissociation constant than the first, the reaction would proceed until the heparin had largely displaced the cephalin. Chargaff *et al.* [1940] found that this heparin-protein compound was inactive as an anticoagulant in thrombin-fibrinogen mixtures, although it possessed full activity in plasma. This indicates that the compound is itself inactive as an anticoagulant; its dissociation constant is, however, fairly high and hence it is completely dissociated in plasma. This appears to be a more reasonable interpretation of the data than the assumption that the heparin-protein compound formed by the reaction is itself an anticoagulant. Washing the precipitate at *p*H 5·0 suppressed dissociation of the heparin-protein in the wash water. The same results were obtained with a cephalin-histone complex, but in this case the heparin-histone compound was inactive in plasma. This was to be expected, since, as reported above, this compound has a low dissociation constant.

It is suggested that the view advanced as to the nature of the reaction of heparin and thromboplastin may be of value in considering all the reactions involved in the clotting system, i.e. that reversible dissociation of the various compounds involved may occur and that when several reactions are possible, that one will occur to the greatest extent which leads to the compound with the lowest dissociation constant. For example, Ferguson has postulated his 'cephalin availability' theory to explain the action of thromboplastin; according to this, thromboplastin serves to transfer cephalin to prothrombin. The idea of dissociable compounds presented here provides a mechanism for such a

theory. Further, trypsin and other proteolytic enzymes, by destroying the protein part of thromboplastin, would increase its dissociation and speed the transfer of cephalin to prothrombin, thus providing a mechanism for the action of trypsin on the compound, the practical significance of which Ferguson has stressed. In the case of those clotting factors which are enzymes, the theory becomes the well-known theory of Michaelis and Menten. From the foregoing it is evident (1) that this reaction of heparin should be considered in experiments requiring its use, and (2) that in studies on blood clotting, heparin activity may be affected by the presence of factors not usually considered.

SUMMARY

Heparin forms stable salts with many proteins. The resulting salt is markedly different in its properties (isoelectric point, solubility, etc.) from the original protein. In solution, these salts show partial dissociation according to the mass law. The dissociation constant of the compound varies with the protein, and the dissociation is markedly affected by *p*H and the presence of other anions and cations. The formation of compounds with a sufficiently low dissociation constant is followed by the loss of biological activity on the part of the anticoagulant. The application of these results to quantitative studies on blood clotting is indicated.

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The Isolation of Behenic Acid from Refined Mona Wax

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Montan wax has been refined by treatment with chromic acid in glacial acetic acid solution [Marcusson & Lederer, 1931]. Mona wax isolated from Irish peat [Reilly & Wilson, 1940] was not changed by this mixture. A sulphuric acid-chromic acid mixture gave 72·5 % of a pale yellow acidic wax. Its properties are compared with those of Mona wax in the following table:

	Mona wax	Refined Mona wax
Acid value	46	149
Saponification value	98	173
Ester value	52	24
Iodine value	30	5·5
M.P.	70°	73·5°

The acids were isolated by the usual procedure [Chibnall, 1934; Reilly & Wilson, 1940] and converted to their methyl esters. By a series of fractional distillations *in vacuo*, the latter were separated into a number of fractions. From one of these, an acid was isolated whose equivalent weight and elementary analysis suggested that it might be behenic acid $C_{22}H_{44}O_2$.

Stohmann & Langbein [1890] found the M.P. of behenic acid, obtained by the reduction of ethyl erucate, to be 77–78°. The value obtained in the present investigation was 77·5°. A more recent determination on the synthetic acid by Francis, Piper & Malkin [1930] gave a value of 80·0°. To determine its identity, the acid was submitted for X-ray crystallographic examination. C. H. Carlisle and D. M. Crowfoot (see below) showed, by measurements of the B and C crystal spacings, that the acid was contaminated with 2·5–10 % of $C_{20}H_{40}O_2$, and probably with traces of a higher acid, $C_{24}H_{48}O_2$.

Holde [1924], from a consideration of the theory of fat formation proposed by Buchner & Meisenheimer [1908], concluded that only normal fatty acids with an even number of carbon atoms can occur in nature. Francis *et al.* [1930] fully supported his conclusion by their work on a number of the higher naturally occurring acids. From an examination of the X-ray photographs they also concluded that a separation into single acids was almost impossible (except when one acid occurred in the mixture in preponderating amounts). Stadler [1933] on the other hand, claims that, after a series of carefully executed fractional distillations *in vacuo*, he isolated, from Bohemia Montan wax, two pure acids containing an uneven number of carbon atoms ($C_{27}H_{54}O_2$ and $C_{29}H_{58}O_2$).

The present investigation was an attempt to verify the last author's results. By using similar distilling apparatus, and adhering to his conditions, the present authors isolated behenic acid ($C_{22}H_{44}O_2$), in an impure condition. These results do not, therefore, support his claim, but are further evidence in favour of the conclusions reached by Francis *et al.* [1930].

EXPERIMENTAL

A total of 190 g. of pure refined mona wax was used.

Saponification and isolation of calcium salts. 50 g. wax were dissolved in 500 ml. benzene and 200 ml. ethanol. 25 g. KOH, in 250 ml. ethanol, were added to the hot solution and the mixture boiled for 3 hr. under reflux. The content of the flask was divided in two and a hot solution of anhyd. $CaCl_2$ (200 g.) was added slowly to each portion while nearly boiling with vigorous shaking. The solution was boiled for 1 hr. to complete the reaction. The Ca salts were filtered off, dried, ground and extracted exhaustively with benzene in a Soxhlet apparatus.

Formation of acids and esterification. The powdered calcium soaps were added in small quantities to warm dilute HCl (1 part acid : 2 parts water) and the mixture boiled till the fatty acids floated in a brown layer on top. When cold, the acids were removed and washed free from mineral acid. Acid value, 185.

The acids were esterified in 20 g. lots. 20 g. acid were refluxed with 1200 ml. methanol and 40 ml. conc. H_2SO_4 for 8 hr. The brown solution of the ester was then decanted from the brown-black residue. After distilling off most of the alcohol the remainder was poured into a large volume of water, when the esters were precipitated. The water was then heated to boiling, when a brown layer of esters collected on top. These were separated and washed free from H_2SO_4. Acid value, 22; saponification value, 178; iodine value, 2·8; M.P. 73°.

An attempt to reduce the acid value by re-esterification failed. The method of Buxton & Kapp [1940], which depends on removing free acid as the K salt, was used, but it reduced the acid value only to 11. Total weight of methyl esters, 115·8 g.

Fractionation. All-glass, electrically heated, fractionation columns, similar to those described by Stadler [1933] and Longenecker [1937], were used. Each consisted of an inner tube packed with Lessing rings. This was surrounded by another tube on which was wound nichrome resistance wire. An outer insulating tube enclosed both heating tube and column. The flask containing the esters was fused to the bottom of the column for each distillation. The heating of the column was controlled by external variable resistances and the temperature measured by an iron-nickel couple inserted between the column and heating jacket. Jantzen & Tiedecke [quoted by Stadler, 1933], in a study of fractionation, found that the boiling-point was not determinate of the course of fractionation. These workers fractionated according to the melting-points of the distillate. This method was used by Stadler [1933] and was adopted in the present work.

Preliminary distillation was undertaken in order to free the esters from any impurities, and to divide them into a higher and a lower fraction. The ester was divided into three portions (two of 40 g., one of 35·8 g.). Each was distilled (2–5 mm.) from a 250 ml. Claisen flask, and the distillate collected by means of a Perkin triangle.

Lower fraction	56·6 g.
Higher fraction	28·9 g.
Total	85·5 g.

Fractional distillation. The lower and higher fractions were placed separately with some glass wool and powdered pumice stone in two 250 ml. Pyrex flasks which were fused to the bottom of a column 47 cm. long and 3 cm. internal diameter. Test tubes fused on to the side arm were used as receivers. Table 1 gives the details of fractionation of the higher and lower fractions. IF is the lower, and IIF the higher fraction.

Table 1. *Fractional distillation of higher and lower ester fractions*

Fraction	Weight g.	M.P. ° C.	Dist. temp. ° C.	Pressure mm.	
IF.	Total 56·6 g.	Distillate 49·41 g.			
(i)	4·10	Liquid	90–125		
(ii)	2·68	Semi-liquid	136	0·10	
1	3·68	31·5	126		
2	1·96	34·0	136		
3	5·70	37·5	138		A.V. 7·4
4	2·33	38·5	146–150		
5	1·59	40·5	157	0·15	
6	1·82	41·0	160		
7	3·84	42·3	166		
8	4·25	42·5	168		
9	2·02	45·0	155		
10	2·67	46·5	160–166		
11	2·98	47·0	163	0·15	
12	3·17	48·5	154		A.V. 7·7
13	4·04	51·5	178		
14	2·58	54·0	186	0·30	
IIF.	Total 28·9 g.	Distillate 26·62 g.			
(i)	2·97	Liquid	156	0·20	
1	4·08	39·5	170		
2	5·09	45·0	172		
3	2·88	49·0	182		
4	4·78	53·0	174		A.V. 1·7
5	3·49	53·5	205		
6	3·33	57·0	210		

The low acid values show that very little decomposition had taken place during the distillations.

Table 2. *Grouping of ester fractions for further distillation*

Group	Fractions combined	M.P. range °C.
I R.	I 9–11, II 2	45·0–47·0
II R.	I 14, II 4, 5	53·0–53·5
III R.	I 12–13	48·5–51·5
F.F.	II R 7–10 III R 2–4	48·8–52·5

Table 3. *Data for distillation of the three main fractions*

Fraction no.	Wt. g.	M.P. °C.	Dist. temp. °C.	Pressure mm.
(*a*) Fraction I R.	Total 12·7 g.		Distillate 12·3 g.	
(i)	0·50	Liquid	92	0·05
1	0·83	41·0	101	
2	0·58	40·5	112·5	
3	1·59	43·0	106	
4	0·60	43·5	109	
5	0·93	45·0	114	
6	1·2	46·0	111	0·01
7	0·87	48·0	112	
8	0·46	47·6	106	
9	0·38	48·5	106–111	
9 A	1·00	49·0	—	
10	1·66	49·5	122–4	
11	0·97	50·5	115	
12	0·73	54·0	135–172	
(*b*) Fraction II R.	Total 10·17 g.		Distillate 9·76 g.	
(i)	0·46	Liquid	92	0·05
1	0·84	48·0	100	
2	0·87	51·5	126	
3	1·69	52·5	129	
4	1·87	54·0–54·6	118	
5	0·40	55·0	113	
6	1·11	56·5–57·2	138	
7	1·04	59·0–59·2	130	0·01
8	1·48	59·0	132	
(*c*) Fraction III R.	Total 5·30 g.		Distillate 4·81 g.	
1	0·35	48·0	98	0·03
2	0·38	49·7	115	
3	0·59	50·2	120	
4	0·74	52·5	117	
5	1·40	53·5	130	
6	0·23	55·0	111	
7	0·65	56·0	104	
8	0·47	56·4	120	

The fractions were divided into groups according to their melting-points and the groups I R to III R were then distilled separately through a smaller column of the same type (26 × 2 cm.). The scheme is clear from Table 2 and the data are given in Table 3.

For the final fractionation, some fractions of the highest weight and shortest temperature range were chosen (Table 4). Fractions I R 7–10 and III R 2–4 were combined and distilled through a third fractionating column. Instead of a flask, a small elongated bulb containing the ester was fused on to the end of the column. The bulb was wound with resistance wire (nichrome no. 15) for heating purposes, and the temperature was measured by means of an iron-nickel couple. This heating arrangement was found to be more satisfactory than the metal bath previously used, and allowed of more careful temperature control.

Table 4. *Data from final fractionation*

Total 5·0 g. Distillate 4·08 g.

Fraction no.	Wt. g.	M.P. °C.	Dist. temp. °C.	Pressure mm.
1	0·27	47·8	93	0·01
2	0·42	48·5	94	
3	0·32	49·0	94	
4	0·36	49·1	94–100	
5	0·33	49·2	100	
6	0·30	49·7	80	0·04
7	0·29	49·7	88	
8	0·25	50·0	90	
9	0·41	50·3	95	
10	0·27	50·2	97	
11	0·25	50·4	100	
12	0·28	50·5	102	
13	0·33	50·8	105	

Fractions nos. 10, 11 and 12 were worked up to obtain the acid. The results are given in Table 5.

Table 5. *Data on isolated behenic acid*

Origin of acid	Quantity mg.	KOH ml.	Normality of alkali	Equiv. wt.	Acid value	M.P. °C.
A	101·4	11·25	0·0261	344·2	162·7	
	100·0	11·00	0·0261	340·0	164·9	77·5
	100·6	11·2	0·0266	336·0	166·8	
	103·2	—	0·0266	342·6	163·8	

A, therefore, consists mainly of behenic acid ($C_{22}H_{44}O_2$) of acid value 165, and equivalent weight 340·35. (Analysis: found 77·23% C, 12·5% H; 77·23% C, 12·7% H. Calc. for 77·6% C, 12·9% H. $C_{22}H_{44}O_2$.)

X-ray Crystallographic Examination of Fatty Acids Obtained from Mona Wax

By C. H. Carlisle and D. Crowfoot

In order to obtain further evidence on the composition of fraction A, it was submitted to X-ray crystallographic examination. For comparison, one of the higher melting fractions was studied in addition.

The results of the X-ray examination are shown in Table 6, together with the most recent data obtained on pure fatty acids by Francis & Piper [1939]. This table confirms the general conclusions already reached as to the main constituents of these fractions, and also shows clearly that neither fraction is pure. Unfortunately no exact X-ray measurements have been made on synthetic binary mixtures of the acids most likely to be present, i.e. $C_{20}H_{40}O_2$, and $C_{22}H_{44}O_2$, or some ternary mixtures of these acids with $C_{24}H_{48}O_2$, and the variations in X-ray spacing of

other mixtures, e.g. of $C_{22}H_{44}O_2$ and $C_{24}H_{48}O_2$, which have been examined [Chibnall, Piper & Williams, 1936], show too many irregularities to be used for direct comparison. Some estimate of the nature of the impurities present may, however, be made by correlating the X-ray measurements with the general observations made by Chibnall *et al.* [1936] on the behaviour of fatty acid mixtures. For this purpose measurements had to be made of both the B and C polymorphic modifications, the X-ray photographs usually being taken of samples pressed lightly on to glass slides.

A. The original preparation, when recrystallized from acetone, showed X-ray lines corresponding to the C modification alone. On recrystallization from benzene and from acetic acid, preparations were obtained, both of which showed lines due to B and C modifications on the same photograph. Such behaviour was noted by Piper and co-workers [Chibnall *et al.* 1936] in the binary mixture containing 2·5–10 % and 80–90 % of the lower constituent. Here the C spacing is slightly lower, the B spacing slightly higher than the best values for pure $C_{22}H_{44}O_2$. Both these types of spacing change may be due to mixture of a small quantity of $C_{20}H_{40}O_2$, say 2·5–10 %, with $C_{22}H_{44}O_2$, but it seems very probable that the fraction still contains traces of other acids, particularly $C_{24}H_{48}O_2$.

III R 5. This fraction, when recrystallized from methanol-ethyl acetate, showed the B spacing alone. While the spacings, both of this, and of the methyl ester, are intermediate between those of $C_{22}H_{44}O_2$ and $C_{24}H_{48}O_2$, the data do not fit very well with the observations that have been made on mixtures of synthetic $C_{22}H_{44}O_2$ and $C_{24}H_{48}O_2$, so that the mixture is presumably more complex.

Table 6. *X-ray measurements*

Acid fraction	M.P. ° C.	B spacing	C spacing	Methyl ester spacing
A	77·5	53·6	47·7	—
III R 5	81·9	56·5	—	61·0
Compare				
$C_{20}H_{40}O_2$	73·5	48·45	44·15	52·3
$C_{22}H_{44}O_2$	79·95	52·95	48·3	57·02

SUMMARY

1. The isolation of behenic acid from refined mona wax is described.

2. The results of X-ray crystallographic examination of the product, by C. H. Carlisle & D. Crowfoot, are reported. They confirm the conclusion that the isolated product consists largely of behenic acid.

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A Study of the Excretion of Organic Antimonials Using a Polarographic Procedure

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Several attempts have been made to determine the rate of excretion of antimony compounds during the treatment of tropical diseases, and so to control dosage and avoid toxic symptoms. The early qualitative tests for the presence of the metal in urine [Di Cristina & Caronia, 1916; Mallardi, 1921] were replaced by quantitative methods based upon the colorimetric determination of antimony as sulphide [Brahmachari, Das & Sen, 1923; Khalil, 1931] or by a modification of the Gutzeit test [Boyd & Roy, 1929]. A summary of this work is given in Table 1. All the quantitative procedures were tedious; the degree of accuracy was low, and the chemical treatment made it impossible to distinguish between tervalent and quinquevalent antimony in the original samples. The subjects were various human patients

and laboratory animals, and a number of different antimony compounds were used, with varying dosages and methods, so that no scale of comparison had as yet been provided. The present work provides such a scale for a number of widely used antimonials which have been injected in comparable doses into the same species of laboratory animal. It also introduces the rapid method of polarography for the direct determination of antimony in both tervalent and quinquevalent forms. A short summary of a few of our results has already been recorded [Goodwin & Page, 1942].

Table 1

Observer	Disease treated	Drug used	Method of assay
Di Cristina & Caronia [1916]	Mediterranean kala-azar	Tartar emetic	Qualitative test (sulphide)
Christopherson & Newlove [1919]	Schistosomiasis	Tartar emetic	Beam & Freak [1919] method presumably
Mallardi [1921]	Mediterranean kala-azar	Tartar emetic; stibacetin	Semi-quantitative modified Marsh test
Renault, Monier-Vinard & Gendron [1922]	Mediterranean kala-azar	Stibacetin	Iodine (?) titration
Brahmachari, Chaudhury, Das & Sen [1924]	Indian kala-azar	Na antimonyl tartrate; urea stibamine	Modified Beam & Freak method
Brahmachari & Sen [1924]	Indian kala-azar	Various antimonyl tartrates	Modified Beam & Freak method
Delius [1925]	Disseminated sclerosis	Antimosan	Marsh test
Boyd & Roy [1929]	Indian kala-azar	Na antimonyl tartrate; Neostibosan	Modified Gutzeit test
Peter [1929]	Schistosomiasis	Fouadin (stibophen)	Not stated
Khalil [1931]	Schistosomiasis	Fouadin	Modified Beam & Freak method
Piazza [1933]	Mediterranean kala-azar	Tartar emetic; Neostibosan	Semi-quantitative colorimetric test (sulphide)
Khalil [1936 *a*, *b*]	Schistosomiasis	Fouadin	Colorimetric test for pyrocatechol said to run parallel with the urine Sb content
Hassan [1937, 1938*a*]	Schistosomiasis	Tartar emetic; Fouadin	Modified Gutzeit test on ashed urine
Weese [1937]	Normal rabbits	Neostibosan; Solustibosan	Not stated
Weese [1938*a*]	Normal dogs	Fouadin	Not stated

The polarographic method of micro-chemical analysis was devised about 20 years ago by Heyrovsky, but it is only comparatively recently that self-contained instruments have become available for routine analytical procedures. Several comprehensive accounts [Kolthoff & Lingane, 1941; Müller, 1941] of the principles of analysis with the dropping mercury electrode have been published, and these should be consulted for practical details of the standard procedures. The general factors to be considered in quantitative work have been reviewed by Kolthoff [1942].

The polarograph has been widely used for the determination of the inorganic constituents of biological materials, but the procedures are frequently tedious, since many organic substances are reducible at the cathode and the materials have consequently to be prepared for analysis by special pretreatments. Such methods have been described for the determination of copper in liver ash by Shoji [1931] and Mandai [1931] and in blood by Roncato & Bassani [1934] and Kamegai [1939*a*, *b*]; for lead in blood by Teisinger [1938], in urine by Reed & Gant [1942], and in both blood and urine by Cholak & Bambach [1941]; for alkali metals in blood by Schweitzer [1936] and in urine by Kemula & Michalski [1939]. The determination of arsenic in biological materials has recently been reported by Bambach [1942]. The lengthy pretreatment is typical of the procedures usually required for the polarographic examination of such materials.

The polarographic determination of antimony was described by Page & Robinson [1942], who demonstrated that in the presence of normal HCl the diffusion current for tervalent Sb is directly proportional to the concentration over the range 0·05–0·0001 % Sb. Tervalent but not quinquevalent Sb in normal HCl has a half-wave potential versus the saturated calomel electrode of −0·15 V., consequently tervalent Sb can be determined directly in the presence of the quinquevalent form.

The determination of Sb in blood and urine is remarkably simple. Blood requires relatively little pretreatment, while for urine the determination is direct. The half-wave potential of tervalent Sb versus the saturated calomel electrode being −0·15 V., its characteristic step appears before those of other reducible constituents of urine. The latter would completely mask small quantities of a substance less readily reducible than tervalent Sb. A similar procedure can be adopted for the determination of bismuth [Page & Robinson, 1942].

METHODS

Technique of polarographic determination of antimony

In order to estimate quinquevalent Sb it must first be reduced by boiling with Na sulphite, and can then be determined in the usual way. For a sample containing both ter- and quinquevalent Sb, the tervalent Sb is first estimated, the sample reduced, and the total Sb determined, the quinquevalent Sb being obtained by difference. These points are illustrated in Fig. 1. Under favourable conditions, the error does not exceed $\pm 2\%$ over the concentration range 10^{-1} to 10^{-3}, $\pm 5\%$ over the range 10^{-3} and 10^{-4}, and $\pm 10\%$ over the range 10^{-4} and 10^{-5} g./100 ml.

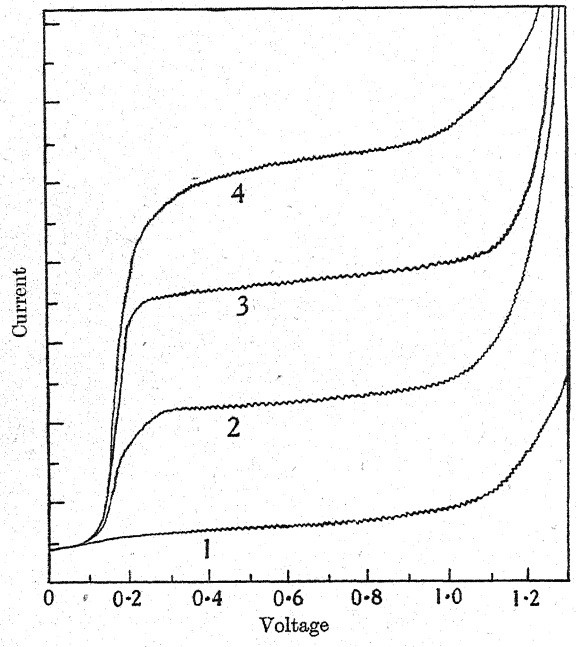


Fig. 1. Antimony polarograms.

(1) 0·0001 *M* sodium antimony^{v} gluconate.
(2) 0·00005 *M* sodium antimony^{v} gluconate } reduced.
(3) 0·0001 *M* sodium antimony^{v} gluconate } reduced.
(4) 0·00015 *M* sodium antimony^{v} gluconate } reduced.

A Cambridge polarograph [Page & Robinson, 1942] was used for this investigation, the small cell being particularly useful, since it required only 5 ml. of solution. The supplementary 2000 μF. electrolytic condenser ensured a sharp polarogram, which facilitated the measurement of small diffusion currents.

All solutions were made normal to HCl and the Sb content adjusted within the range 0·05–0·001 %. The instrument was calibrated by recording on the same photographic paper as the unknown, the polarogram of a standard Sb solution. So long as the same capillary was employed and the mercury column maintained at a constant height, there was very little difference in the standardization during the course of several months.

Polarographic estimation of antimony in urine

Collection of urine. It was found that metal metabolism cages were unsatisfactory in this work, as small quantities of metals derived from the cage interfered with the Sb determination (especially in polarographic estimations). An all-glass metabolism cage was therefore devised, in which the animals rested upon a grille of glass rods, and the urine came into contact with nothing but glass. Groups of ten 20 g. mice were used in the cages, and the bladder of each one emptied by gentle pressure on the abdomen at the end of every collection period. The cages were washed down with 50 ml. of distilled water, a sufficient quantity to ensure recovery of 80–90 % of the Sb in the cages, without undue dilution of the sample.

Preparation of urine samples. (*a*) *For estimation of tervalent antimony.* The urine collected in the glass metabolism cage was made normal to HCl with strong acid and a sample examined immediately on the polarograph. The operation was performed as rapidly as possible after the addition of the acid as we have shown (*vide infra*) that many quinquevalent Sb preparations are hydrolysed to the tervalent state on standing with HCl.

(*b*) *For estimation of total antimony.* 5 ml. of the urine sample after treatment with HCl were boiled for 10 min. with about 0·05 g. Na sulphite, cooled, made up to 5 ml. with a few drops of strong HCl, and the total Sb determined. It is essential to boil off all the SO_2, since Na_2SO_3 in *N* HCl yields a step of its own, which seriously interferes with the Sb step.

Polarographic estimation of antimony in blood

(*a*) *Estimation of tervalent antimony.* It is essential that the necessary treatment of the blood before polarography should not change the valency state of the Sb present, and that the reagents used should not interfere with the polarographic step for Sb. A preliminary investigation on the influence of protein precipitants was therefore necessary.

Whole blood, serum or plasma cannot be used directly, since on addition of HCl the protein is precipitated and carries down a considerable proportion of the Sb with it. Trichloroacetic acid, tungstic acid and zinc hydroxide interfere with the polarographic step. The only suitable protein precipitant is sulphosalicylic acid, which has been used by Brdicka [1938] and also by Waldschmidt-Leitz & Mayer [1939] for the removal of protein from

serum, in their investigations on the polarographic detection of cancer. However, some loss of Sb occurs with whole blood and sulphosalicylic acid, even if the blood is diluted to 1 in 20, but the loss with plasma is considerably less, due to the smaller amount of protein precipitated from it.

Colorimetric estimation of antimony in urine

A modification of Clarke's method [1928] was used. The urine was boiled with copper foil in the presence of HCl, the deposited Sb dissolved from the foil with Na_2O_2 and water, and any copper precipitated from solution with H_2S. The filtered solution containing the Sb was acidified with H_2SO_4, evaporated to small bulk, and then treated with KI, aqueous pyridine and SO_2 in the presence of gum acacia as a protective colloid. The Sb in the clear yellow colloidal solution of the complex iodide was estimated with a Hilger 'Spekker' absorptiometer, calibrated with standard tartar emetic solutions.

Table 2. *Recovery of antimony after treatment with sulphosalicylic acid*

	1 in 10 dilution			1 in 20 dilution		
	Sb^{III} in solution g. $\times 10^{-4}$/100 ml.		Recovery of Sb^{III}	Sb^{III} in solution g. $\times 10^{-4}$/100 ml.		Recovery of Sb^{III}
Sample	Found	Added	%	Found	Added	%
1. Citrated blood	40·4	59·0	70	24·8	32·2	80
2. Plasma	52·0	59·0	90	—	—	—
3. Water	59·2	59·0	100	32·4	32·2	100
4. Plasma	68·8	64·9	107	39·4	35·4	112
5. Corpuscles	2·5	51·3	5	—	—	—
6. Plasma	—	—	—	36·9	35·4	105
7. Corpuscles	—	—	—	4·5	29·0	15

Table 2 shows the percentage recovery of tervalent Sb from citrated blood and plasma to which known amounts of stibophen were added. The same quantity of stibophen was added to nos. 1, 2 and 3. Nos. 4 and 5 were obtained by centrifuging a sample of no. 1 immediately after the addition, while nos. 6 and 7 were obtained after the blood had been standing for 6 hr.

The technique finally adopted was as follows: 1 ml. of the citrated blood was mixed with 19 ml. of distilled water in a centrifuge tube. 2 ml. of 20 % sulphosalicylic acid were added and the tube spun for 15 min. 5 ml. of the clear supernatant liquid were pipetted off, treated with 1 ml. of strong HCl, made up to 10 ml. with distilled water and examined immediately on the polarograph. The recovery of tervalent Sb was about 80 %.

(*b*) *Estimation of total antimony.* 1 ml. of citrated blood was pipetted into 7·5 ml. of 10 % HCl and refluxed in a 50 ml. flask on a sand-bath for 2 hr., during which time the contents of the flask became clear except for a few brownish specks. After cooling, filtering and diluting to 20 ml., the solution was ready for the polarograph.

In the course of this process, all the Sb was reduced to the tervalent form, whatever had been its state in the original blood. The recovery was good, amounting to over 90 % of the theoretical value when tested upon blood containing known amounts of stibophen or Na antimony^{v} gluconate.

(*c*) *Distribution of antimony between corpuscles and plasma.* When the plasma was separated from a sample of blood to which Sb had just been added, the recovery was found to be more than 100 %. The corpuscles, on the other hand, contained practically no Sb, indicating that little had been absorbed. After standing for 6 hr., the concentration in the corpuscles was still very much lower than that in the plasma.

RESULTS

The rate of urinary excretion of organic antimonials by laboratory animals

(1) *Preliminary work using the colorimetric method.* Boyd & Roy [1929] studied the excretion of 'neostibosan' (diethylamine *p*-aminophenylstibonate) after intravenous and intramuscular injection into kala-azar patients, and showed that the initial rate of excretion of Sb in the urine was very high, about 20 % of the dose being recovered in the first 2½ hr. Stibamine glucoside ('neostam') is a quinquevalent compound of the same type as neostibosan, and its rate of excretion by mice was determined to see how it compared with that substance. A few tests were made on rats and hamsters, and certain other Sb compounds were also used. The results in Table 3 are a useful check on the more accurate polarographic determinations recorded in the following section (*b*).

(2) *Results using the polarograph.* The excretion of the following tervalent and quinquevalent antimonials was investigated:

Tervalent. K antimonyl tartrate. Stibophen ('Fouadin')-Na antimony^{III} *bis*-pyrocatechol-3:5-disulphonate. 'Anthiomaline'-Li antimony^{III} thiomalate.

Quinquevalent. (*a*) ('*emetic type*'). Na Sb^{v} gluconate (= 'Solustibosan'?).

Table 3. *The excretion of antimony by laboratory animals (colorimetric method)*

Animals	Drug	Dose mg. Sb/kg.	Method of injection	% dose excreted in urine (hr.) 0–1	1–2	2–4	4–6	6–24	24–48	48–72	72–96	% dose excreted totally in 6 hr.	24 hr.
Mice (groups of 10)	Stibamine glucoside (Sb^{v})	62·5	Subcut.	11·4	6·9	6·6	6·1	13·7	—	—	—	31·0	44·7
				15·2	14·5	8·0	6·0	8·2	—	—	—	43·7	51·9
				8·3	5·1	5·3	8·1	2·8	—	—	—	26·8	29·6
			Intrap.	13·1	13·3	8·2	8·9	9·8	—	—	—	43·5	53·3
				16·6	4·8	6·9	4·0	7·7	—	—	—	32·3	40·0
				25·4	2·3	11·4	6·9	8·3	—	—	—	46·0	54·3
			Intrav.	35·4	6·9	5·1	2·7	5·5	—	—	—	50·1	55·6
				32·7	10·2	3·2	2·3	5·1	—	—	—	48·4	53·5
				23·0	12·3	4·0	13·6	4·8	—	—	—	52·9	57·7
,,	Stibophen (Sb^{III})	41·0	Subcut.	32·4	3·8	13·0	3·2	5·4	—	—	—	52·4	57·8
				41·0	5·1	3·2	1·1	—	—	—	—	50·4	—
				40·4									
			Intrap.		9·6	2·2	4·1	5·7	—	—	—	52·2	56·3
				14·0	12·8	17·6	6·6	7·8	—	—	—	51·0	58·8
				26·0	7·8	4·9	2·3	4·3	—	—	—	41·0	45·3
			Intrav.	66·0	6·5	2·5	1·7	5·4	—	—	—	76·7	82·1
,,	Tartar emetic (Sb^{III})	6·0	Intrap.	8·0		3·0	2·8	16·0	5·0	—	—	13·8	29·8
Rats (groups of 6)	Stibamine glucoside (Sb^{v})	33·3	Intrap.			45·0			1·4	0·6	0·4	—	45·0
						41·0			1·7	0·6	0·3	—	41·0
						43·0			7·0	2·5	1·2	—	43·0
						41·0			5·0	1·6	1·2	—	41·0
Hamsters (mean values for 10 exps. with single animals)	Solustibosan	160·0	Subcut.			61·2			3·1	3·4	—	—	61·2
	Na Sb^{v} gluconate	160·0	Subcut.			56·7			2·1	1·6	—	—	56·7

(*b*) (*derivatives of phenylstibonic acid*). Stibamine glucoside ('Neostam')-N-glucoside of Na *p*-aminophenylstibonate. 'Neostibosan'-diethylamine *p*-aminophenylstibonate. 'Urea stibamine'-*s*-diphenylcarbamide-4:4′-distibonic acid and other compounds [Gray, Trevan, Bainbridge & Attwood, 1931]. 'Stibacetin'-Na *p*-aminophenylstibonate.

Na antimony^{v} gluconate, stibophen and tartar emetic were given by the subcutaneous, intraperitoneal and intravenous routes, the first two compounds in both large and small doses. The small dose level of about 3·5 mg. Sb/kg. of mouse was the equivalent of about twice the ordinary therapeutic human dose. Smaller doses were not used, as the limit of sensitivity of the method was reached in the case of the 6–24 and 24–48 hr. samples.

The large dose level of 35 mg. Sb/kg. was, of course, much larger than is ever given in clinical practice, and with some of the compounds given (tartar emetic, anthiomaline, urea-stibamine) was impracticable owing to its toxic effect. An intermediate dose was given in most of these cases. The full range of results is given in Table 4 and some examples are shown graphically in Figs. 2, 3 and 4.

A single experiment was made using a rabbit, to determine the rate of excretion of Na antimony^{v} gluconate at shorter intervals than 1 hr. after the injection. The animal was anaesthetized with nembutal, and a cannula tied into the left ureter. A dose of the compound containing 10 mg. Sb was injected into the left jugular vein, and the urine collected at 15 min. intervals, the cannula being washed out at each collection. Quantities of 4·8, 5·1, 4·9 and 4·6 % of the Sb in the dose injected were recovered during the first hour—19·4 % in all.

For comparison with the excretion rates, the total blood Sb levels in mice given the largest doses (35 mg. Sb/kg.) of stibophen and Na antimony^{v} gluconate by the three routes of injection were determined at intervals. Mice weighing 20–25 g. were used, and groups of four killed from time to time. The heart blood from all members of one group was pooled, and 1 ml. taken for a determination (Table 5).

(3) *Discussion.* The excretion results show general agreement with earlier workers. The large doses of stibophen and Na antimony^{v} gluconate were excreted at a higher initial rate than the small doses, especially after intravenous injection. After the first hour, the rates of excretion at the two dose levels did not differ very greatly. By the end of 48 hr., almost the same total percentage of the dose

Table 4. *The excretion of antimony by mice*

Drug	Dose mg. Sb/kg.	Method of injection	% dose excreted in urine (hr.) 0–1	1–2	2–3	3–4	4–5	5–6	6–24	24–48	% dose excreted totally in 6 hr.	48 hr.
Tervalent compounds												
Stibophen	3·9	Subcut.	10·5	9·5	4·0	5·5	2·5	4·0	16·0	11·5	36·0	63·5
	3·6	Intrap.	9·5	6·0	4·0	5·5	5·0	5·5	19·5	11·0	35·5	66·0
	3·6	Intrav.	18·0	8·0	9·5	7·5	6·0	7·0	14·0	12·0	56·0	82·0
	33·5	Subcut.	21·8	10·4	2·9	2·3	5·4	5·4	11·0	4·2	48·2	63·4
	33·5	Intrap.	25·4	7·5	6·7	7·6	6·3	4·9	9·4	4·4	58·4	72·2
	30·4	Intrav.	46·3	5·9	3·3	2·2	6·4	1·7	7·1	4·3	65·8	77·2
Tartar emetic	3·8	Subcut.	10·0	5·0	4·5	6·0	4·5	4·0	15·0	8·5	34·0	57·5
	3·8	Intrap.	5·5	6·0	6·5	4·5	4·5	4·0	14·0	12·0	31·0	57·0
	3·7	Intrav.	8·5	8·5	7·5	8·0	5·5	8·5	15·0	7·0	46·5	68·5
	10·9	Intrap.	5·0	2·0	9·5	8·0	8·0	4·0	12·0	7·5	36·5	56·0
*Anthiomaline	3·5	Intrav.	9·0	10·0	4·0	1·0	1·0	1·0	14·0	10·0	26·0	50·0
Quinquevalent compounds												
Na antimony gluconate	3·5	Subcut.	11·0	11·0	7·0	4·5	2·0	1·0	6·5	8·0	36·5	51·0
	3·5	Intrap.	11·5	11·5	5·5	3·0	1·5	2·5	10·0	8·0	35·5	53·5
	3·5	Intrav.	20·0	11·5	7·5	8·0	3·5	3·5	6·5	6·5	54·0	66·5
	32·5	Subcut.	38·0	12·0	4·0	3·5	2·5	2·0	4·0	3·5	62·0	69·5
	32·5	Intrap.	23·0	6·0	8·0	6·0	1·5	1·0	6·5	4·5	45·5	56·5
	32·5	Intrav.	40·5	10·5	2·5	1·5	5·5	1·5	4·0	2·5	62·0	68·5
*Stibamine glucoside	3·5	Intrav.	15·0	15·0	10·0	5·0	5·0	2·0	7·0	5·0	52·0	64·0
	33·3	Intrav.	32·0	12·0	5·0	3·0	2·0	1·0	4·0	2·0	55·0	61·0
*Neostibosan	3·6	Intrav.	14·0	11·0	7·0	0·0	4·0	3·0	7·0	4·0	39·0	50·0
	25·6	Intrav.	22·3	10·2	8·2	1·6	1·0	2·5	6·4	3·1	45·8	55·3
*Urea-stibamine	3·5	Intrav.	18·0	11·0	9·0	4·0	3·0	1·0	4·0	6·0	46·0	56·0
	11·2	Intrav.	13·7	16·5	8·0	6·2	1·8	5·3	11·6	3·1	51·3	66·0
*Stibacetin	4·2	Intrav.	14·0	11·0	13·0	7·0	5·0	2·0	10·0	5·0	52·0	67·0
	34·3	Intrav.	28·2	7·7	11·2	6·1	5·0	1·0	2·9	3·0	59·2	65·1

* Tests made on groups of 10 mice. All the other figures are means of two experiments, each using a group of 10 mice.

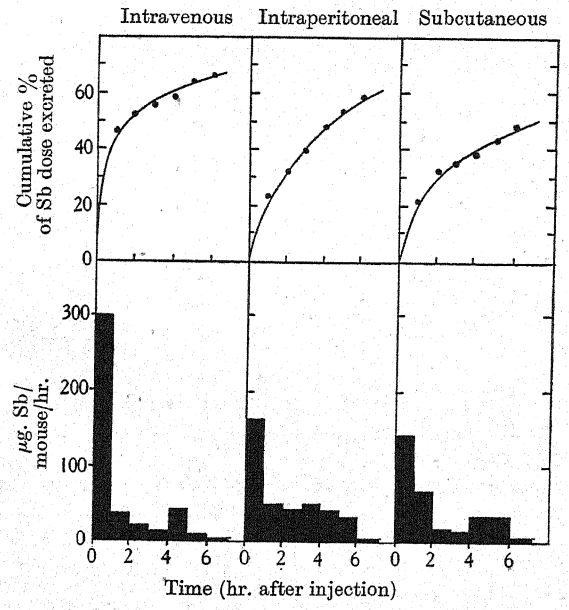


Fig. 2. The excretion of stibophen by mice. (Dose approx. 35 mg. Sb/kg.)

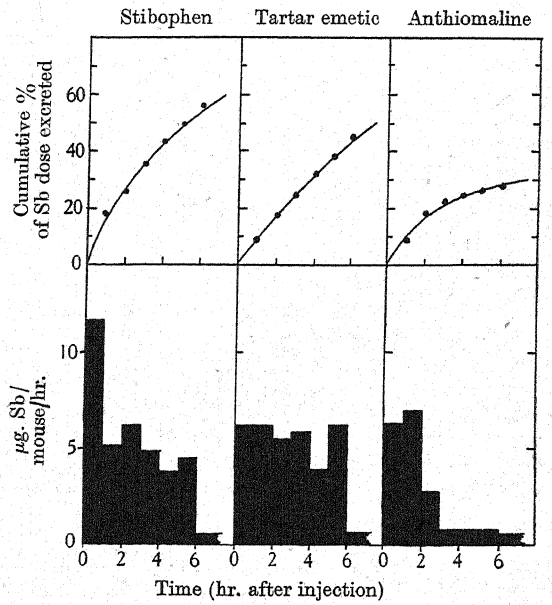


Fig. 3. The excretion of tervalent antimony compounds after intravenous injection in mice. (Dose approx. 3·5 mg. Sb/kg.)

given had been excreted at both dose levels, the excretion rate during the 6–48 hr. interval being proportionately greater with the small doses. Weese [1938*b*] suggests that the large doses flood the whole body, and the 'overflow' is excreted very rapidly, the amount of the overflow depending upon the amount injected. Tartar emetic shows a fairly constant rate of excretion over the first 6 hr., but the rate is considerably greater with mice than has been reported in human clinical cases [Brahmachari *et al.* 1924; Boyd & Roy, 1929; Hassan, 1937]. The most rapidly excreted antimonial in the quinquevalent group was Na antimony^{v} gluconate, and then in order came stibacetin, stibamine glucoside, urea-stibamine and neostibosan—though there was little to choose between them. Stibophen in the tervalent group was not a great deal slower than Na antimony^{v} gluconate, but tartar emetic was much slower, and anthiomaline slower still.

Intravenous injections were in every case the most rapidly excreted. Intraperitoneal and subcutaneous doses did not differ greatly in their rates of excretion.

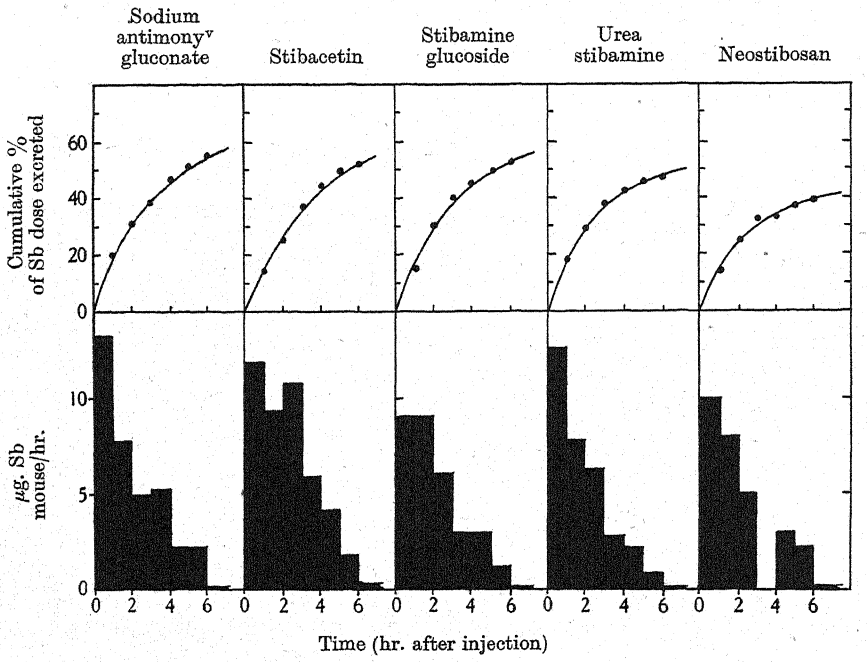


Fig. 4. The excretion of quinquevalent antimony compounds after intravenous injection in mice. (Dose approx. 3·5 mg. Sb/kg.)

Table 5. *Blood antimony in mice given injections containing 35 mg. Sb/kg.*

Groups of four animals were killed at each time period, and the blood pooled

Drug	Method of injection	Blood antimony (mg./100 ml.) (min. after injection) 0	5	10	15	20	30	60	120	180	240	300
Stibophen (Sb^{III})	Subcut.	0·6	1·7	2·0	—	2·0	1·7	1·4	1·1	0	0	0
	Intrap.	0·6	—	2·6	2·0	2·2	2·0	1·6	1·2	0·6	0	0
	Intrav.	4·8	0·7	1·7	—	1·7	1·7	1·1	0·3	0	0	0
Na antimony^{v} gluconate (Sb^{v})	Subcut.	0·3	0·8	1·3	—	1·4	1·1	0·5	0	0·3	0	0
	Intrap.	0·2	1·0	1·2	—	1·0	1·2	0·8	0·4	0	0	0
	Intrav.	7·8	4·0	4·0	—	2·3	1·2	1·0	0·8	0	0	0

The rabbit experiment showed an almost constant rate of excretion during the first hour after a dose of Na antimony^{v} gluconate. If the other kidney is assumed to have excreted at the same rate, about 40 % of the dose was eliminated in the first hour, a result which agrees with the mouse experiments.

From Table 5 it will be seen that in the course of about 3 hr., all measurable amounts of Sb had disappeared from the blood stream. It is interesting to see how efficient the absorption of the two

compounds investigated appears to be. In the time taken to kill a mouse and remove blood from the heart, appreciable quantities of Sb reached the blood stream after subcutaneous and intraperitoneal injection.

The excretion of antimony by human subjects

The polarographic method was applied to the determination of Sb excretion in human subjects. Doses were given intravenously or intramuscularly, and the urine and the capillary or venous blood collected at intervals. Polarographic measurements were much easier than with mice, owing to the larger amounts of Sb present. The results are shown in Tables 6 and 7 and Fig. 5, and though the number of experiments is small, it will be seen that in the case of Na antimony^{v} gluconate, the results correspond to the values for mice fairly closely. With stibophen, however, the rate of excretion is rather slower than in mice, though it should be noted that the small dose given to the mice was 5 times the equivalent of the largest human dose (subject 2). Individual variations were marked.

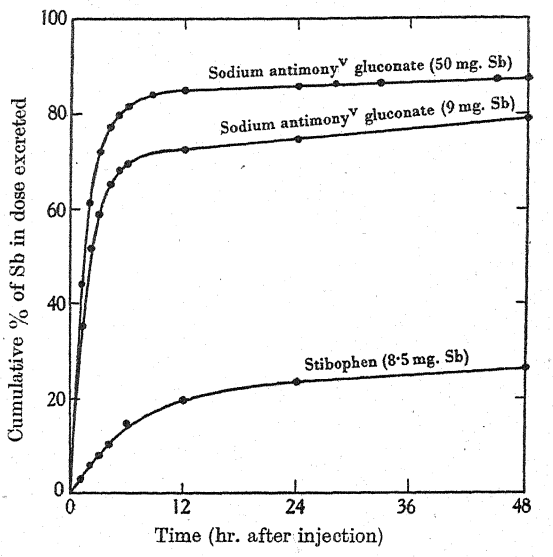


Fig. 5. The excretion of sodium antimony^{v} gluconate and stibophen by human subject (1).

Table 6. *The excretion of antimony by human subjects*

Drug	Dose mg./Sb	Method of injection	Subject no.	% dose excreted in urine (hr.) 0–1	1–2	2–3	3–4	4–5	5–6	6–12	12–24	24–48	% dose excreted totally in 6 hr.	24 hr.
Stibophen (Sb^{III})	42·5	Intrav.	2	8·1	1·4	1·6	0·8	1·0	1·0	2·6	2·8	5·4	13·9	24·7
	8·5	,,	2	6	3	2	1	2	2	2	1	1	16	20
			1	3	3	2	2	5		5	3	4	15	27
	8·5	Intramusc.	4	3	1	2	1	2	2	4	2	1	11	18
			5	11	5	3	2	2	2	2	4	4	25	35
Na antimony^{v} gluconate (Sb^{v})	100	Intrav.	2	26·7	8·2	7·9	5·8	4·7	3·9	7·9	7·8	3·5	57·2	76·4
			3	17·5	15·1	8·9	6·1	7·0	2·4	20·4		—	57·0	77·4
	50	,,	1	44·0	17·6	10·4	5·0	3·0	1·7	2·7	1·0	2·5	81·7	87·9
			6	43·6	17·4	8·6	7·2	4·7	2·2	4·0	2·3	2·4	83·7	92·4
			7	38·8	17·2	8·4	5·9	2·4	3·0	2·8	1·9	2·5	75·7	82·9
	9·0	,,	1	35·4	15·8	8·3	5·9	2·0	1·9	3·1	2·3	5·1	69·3	79·8
Stibamine glucoside (Sb^{v})	50	,,	3	21·0	9·0	6·0	4·0	3·0	3·0	—	—	—	46·0	—

Table 7. *Blood antimony in human subjects*

Drug	Dose mg./Sb	Method of injection	Subject no.	mg. antimony/100 ml. blood (hr.) 0·3	0·5	0·6	1·0	2·0	3·0	4·0	5·0	6·0	24·0
Na antimony^{v} gluconate (Sb^{v})	100	Intrav.	2	1·0	—	0·6	—	0·5	0·3	0	0	0	0
			3	—	—	—	1·5	1·1	0·8	0·3	0·4	—	0
	50	,,	1	1·3	—	0·4	0·3	0·3	—	0	—	0	0
			7	—	1·3	—	0·4	—	—	0·1	—	—	—
Stibamine glucoside	50	,,	3	—	—	—	1·6	—	0·8	—	—	—	—

Subject (1) Normal male, 27.
(2) Normal male, 27.
(3) Male, 24, under treatment for Mediterranean kala-azar.
(4) Normal male, 19.
(5) Normal male, 20.
(6) Normal male, 26.
(7) Male, 25, although suffering from renal stones, is at present in good health.

A dose of Na antimony^{v} gluconate was much more rapidly excreted than one of stibophen containing an equivalent amount of Sb. Hassan [1937] published some results about stibophen excretion, but subsequently corrected these [1938*a*], as it was found that a considerable proportion of the Sb in the drug used in the earlier experiments had become oxidized to the quinquevalent state. Our results agree with those given in Hassan's first paper, though the drug we used was completely free from quinquevalent Sb. It is perhaps worth noting that the 'rise' in excretion rate reported by Hassan after 24 hr. is due to the fact that the period of collection of urine was longer than for the earlier samples. If the excretion *per hour* is calculated, the results lie upon a steadily falling curve.

The influence of the valency of injected antimony on its metabolic fate

The low toxicity of the quinquevalent compounds must be due, in part at least, to their high rates of excretion. Their therapeutic advantages over the tervalent compounds in the treatment of kala-azar have never been satisfactorily explained. A direct leishmanicidal action is not generally favoured, owing to the low toxicity of the compounds to mammals, and it is unlikely that mere stimulation of the host's reticulo-endothelial system could account entirely for their activity.

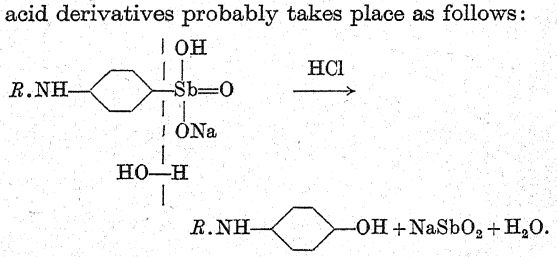
It has therefore been assumed by analogy with the quinquevalent arsenic compounds that reduction takes place in the body to the tervalent state, the stibinoxide grouping (R—Sb=O) being parasiticidal. Since it has been shown above that the polarographic method will distinguish between tervalent and quinquevalent Sb, this method seemed to offer a new approach to the problem.

Brahmachari *et al.* [1924] showed that although the initial excretion of urea-stibamine was rapid, the rest of the dose came away slowly at about the same rate as a dose of tartar emetic, and advanced this slender evidence as proof that the slowly excreted fraction of the quinquevalent compound had been reduced. Bock [1927] thought to show from the results of toxicity tests that the Sb of stibophen was oxidized to the quinquevalent state and so detoxicated. Before the application of the polarograph to the problem, one of us had shown that it was very unlikely that any change in valency had occurred in the initially excreted Sb. The 24 hr. urine collected from groups of 10 mice injected with stibophen or Na antimony^{v} gluconate was passed through a Seitz filter, and the Sb content determined colorimetrically. Doses of the excreted Sb were then injected into mice infected with *Trypanosoma equiperdum*, using animals dosed with fresh solutions as controls. No trypanocidal activity at all was observed with Na antimony^{v} gluconate or with the corresponding urine, but with stibophen the activity at three dose levels was almost identical with that of urine containing the equivalent amounts of Sb. As Na antimony^{III} gluconate has a marked trypanocidal action, and the quinquevalent analogue of stibophen no action at all, it appears that no valency change could have taken place.

By means of the polarograph, it could easily be demonstrated that up to the sixth hour no measurable amounts of Sb of changed valency were excreted either in animals or in man. Also, by precipitating the protein in mouse blood with sulphosalicylic acid, it was shown that the Sb in the clear solution was unchanged in valency after subcutaneous or intravenous injection of Na antimony^{v} gluconate. It was, however, more difficult to be sure of the fate of the Sb remaining in the tissues after the first few hours of excretion. The amounts of substance in every case were very small, and it was difficult to be sure that any valency change observed had occurred in the body, and not during subsequent treatment.

The presence of HCl is essential for these polarographic estimations, and this introduces certain limitations, e.g. it was found that when quinquevalent compounds were treated with normal HCl, hydrolysis took place with the formation of tervalent Sb. Thus with 0·02 % solutions of stibamine glucoside, neostibosan, urea-stibamine and stibacetin, about 30–50 % of the Sb was recorded as tervalent on the polarogram. The hydrolysis took place immediately upon addition of the acid, and no appreciable change was observed on standing for 1 hr. In the case of Na antimony^{v} gluconate however, the reduction was only very slight—about 1–2 % with a 0·02 % solution, and very much less with more concentrated solutions. This is presumably because, unlike other quinquevalent antimonials, the Sb is here joined to the carbon through the oxygen atom, and not directly.

We are grateful to Mr W. H. Gray of the Wellcome Chemical Research Laboratories for the suggestion that the hydrolysis of the *p*-aminophenylstibonic acid derivatives probably takes place as follows:



We have shown that treatment with HCl, followed by neutralization with NaOH, doubles the toxicity of stibamine glucoside as compared with the untreated substance to which an equivalent amount of NaCl had been added. This is further

evidence that the toxic tervalent compound had been produced.

Our choice of a quinquevalent compound for the investigation of valency changes was therefore limited to Na antimony^{v} gluconate, which had to be free from any tervalent Sb, since the presence of as little as 0·1 % of tervalent Sb has been found to be associated with increased toxicity.

Another source of difficulty when dealing with the valency state of Sb excreted in 24 hr. samples of urine, was the change which occurred on standing.

Table 8. *The effect of bacterial growth in urine containing stibophen equivalent to 0·0014 % Sb^{iii}*

Time in hr. ...	26	52	71	95
% of Sb^{III} in test sample (no HCl)	90	80	73	63
% of Sb^{III} in control (+HCl)	99	100	—	—
Time in hr. ...	124	148	169	
% of Sb^{III} in test sample (no HCl)	56	45	34	
% of Sb^{III} in control (+HCl)	—	—	103	

The Sb in stibophen, for example, becomes progressively oxidized to the quinquevalent form if bacterial growth is not prevented. Table 8 shows the effect of unrestrained bacterial activity on urine containing a known amount of stibophen. Urine was, therefore, always collected from the metabolism cage in vessels containing HCl, since this favours reduction, as well as preventing bacterial growth.

By a series of observations, we have satisfied ourselves that a small proportion of injected Sb is reduced in the body. The evidence for this is as follows:

(*a*) In vitro *experiments*. Table 9 shows the effect of incubation at 37° of normal rabbit blood containing Na antimony^{v} gluconate equivalent to 0·001 g./100 ml. of Sb. Samples were withdrawn from time to time, treated with sulphosalicylic acid to remove the protein, and the Sb content determined.

It will be seen that there is a small but significant increase in the proportion of tervalent Sb present after 72 hr. The tervalent Sb recorded in the fresh solution is due to the slight reduction by HCl.

Table 10 shows the effect of incubating small quantities of Na antimony^{v} gluconate with living chick-embryo tissue in serum Tyrode. The tissue cultures were of the type used for the routine preparation of yellow-fever virus, and were kindly provided by Dr F. O. MacCallum. Control cultures which had been killed by heating were incubated at the same time, and although the differences shown in Table 10 are small, there was a distinctly larger amount of tervalent Sb in the living than in the dead tissue cultures. A similar small effect was observed upon incubating with liver slices in Tyrode solution.

When a known amount of Na antimony^{v} gluconate ($\equiv$ 2 mg. Sb^{v}) was incubated with an emulsion of mouse liver (2 g. in 20 ml. saline) 8 % of the Sb was reduced in 3 hr., and 9 % in 24 hr. With an emulsion of kidney (0·5 g. in 20 ml. saline) about 4 % was reduced in 24 hr. No *in vivo* trypanocidal activity could be detected in the incubated solutions, and it appears therefore that the reduction of Sb by living tissues is less rapid than the reduction of quinquevalent As [Levaditi, 1909 *a*, *b*; Lourie, Murgatroyd & Yorke, 1935].

(*b*) *Excretion experiments*. It is in the later fractions of urine collected after a dose of Sb that any reduced substance must be sought. Since, when mice are used, the absolute amounts present in these fractions are very small, we turned our attention to human experiments. The urines collected from subjects 2 and 3 showed a considerable amount of tervalent Sb, but as they had been allowed to stand a considerable time with HCl, this evidence was worthless. The urine samples from subject 1, however, were acidified and polarographed immediately

Table 9. *The effect of incubating at 37° rabbit blood containing Na antimony^{v} gluconate equivalent to 0·001 % Sb^{v}*

		% antimony recovered (hr. after incubation)										
		0	0·5	1·0	2·0	3·0	4·0	5·0	6·0	24·0	72·0	96·0
Experimental (blood)	Sb^{III}	6	7	6	7	—	7	—	5	9	10	15
	Total Sb^{III} and Sb^{v}	95	—	—	—	—	98	—	—	—	105	100
Control (water)	Sb^{III}	6	—	6	—	4	—	4	—	4	6	6
	Total Sb^{III} and Sb^{v}	100	—	—	—	93	—	—	—	—	108	95

Table 10. *The effect of incubation of Na antimony^{v} gluconate with living chick embryo tissue*

Culture	Conc. of total Sb in culture mg./100 ml.	% Sb recovered as Sb^{III}
Experimental (living tissue culture)	0·48	4·2
	0·80	6·3
	1·26	11·1
	1·40	10·7
Control (culture killed by heat before addition of Sb)	0·68	0
	0·80	0
	1·09	5·5
	1·40	7·1

on collection, so that any tervalent Sb present must have been produced in the body.

The proportions of tervalent to total Sb present are shown in Table 11. The fractions collected at all periods except the 12–24, 28–33, and 33–45 hr. collections were from a single emptying of the bladder, and they show a considerable, and increasing, proportion of tervalent Sb.

Table 11. *Antimony excretion by subject 1 after a dose of Na antimony^{v} gluconate equivalent to 50 mg. Sb^{v}*

Time of collection hr.	Vol. of urine ml.	Total Sb excreted mg.	Sb^{III} excreted mg.	Sb^{III} %
0– 1	700	22·0	0·49	2·2
1– 2	193	8·8	0·10	1·1
2– 3	212	5·2	0·16	3·1
3– 4	68	2·5	0·19	7·6
4– 5	62	1·5	0·04	2·7
5– 6	42	0·88	0·01	1·1
6– 9	172	1·16	0·18	15·5
9–12	312	0·19	0·06	32
*12–24	714	0·50	0·50	100
24–28	360	0·25	0·14	56
*28–33	504	0·55	0·30	55
*33–45	408	0·41	0·28	68
45–48	84	0·06	0·03	50

* Collected in several fractions, and allowed to stand until end of collection period.

(*c*) *Antimony in the body organs.* We have confirmed the findings of other workers [Boyd, Napier & Roy, 1931; Hassan, 1938*b*] that Sb is concentrated in the liver, and is present in only very small quantities in the other organs if the animals are not killed immediately after injection. The livers of treated rabbits and mice contained easily measurable amounts of Sb determined by the method for total Sb in blood as is shown in Table 12. In order to determine the valency of the liver Sb, weighed amounts of liver from animals which had received a series of doses of Na antimony^{v} gluconate were ground with sand and ice-cold saline as soon as possible after removal from the body. We did not hope by this method to extract all the Sb present, but merely to determine the valency state. A control experiment was made in each case by grinding up normal liver with a known amount of Na antimony^{v} gluconate and saline. The protein was precipitated with sulphosalicylic acid, the solution was centrifuged, and HCl added to the supernatant solution which was polarographed at once. It will be seen from Table 12 that far more tervalent Sb was recovered from the livers of the injected animals than from the control experiments. We have no information as to the fate of the rest of the Na antimony^{v} gluconate molecule.

Table 12. *The recovery of antimony from the livers of animals injected with Na antimony^{v} gluconate*

Animal		Sb present mg./g. liver	Recovered Sb^{III} % of total
Rabbit given 11 doses of Na antimony^{v} gluconate (170 mg. Sb in all)	Test	0·01	100
	Control (normal liver + Sb^{v})	0·02	1
Nine mice given 10 doses of Na antimony^{v} gluconate (180 mg. Sb in all)	Test (9 livers)	0·03	25
	Control (10 normal livers + Sb^{v})	0·023	4

SUMMARY

1. The rapid polarographic method of analysis has been used for the determination of antimony in biological materials.

2. The rates of urinary excretion of a series of organic antimonials (tartar emetic, 'anthiomaline', stibophen, stibacetin, stibamine glucoside, 'neostibosan', 'urea-stibamine', sodium antimony^{v} gluconate) have been measured after injection into laboratory animals. Three compounds (stibophen, stibamine glucoside and sodium antimony^{v} gluconate) have been tested in a small number of human subjects.

3. It has been shown that about 30–40 % of the antimony content of an injection of stibophen or of one of the quinquevalent compounds is excreted by mice in the urine during the first 1 or 2 hr. The subsequent excretion rate is less rapid. Small doses are excreted more slowly than large ones. Tartar emetic and 'anthiomaline' do not show such a high initial outflow. In the human subjects, a dose of Na antimony^{v} gluconate was excreted more rapidly than one of stibophen containing the same amount of antimony.

4. The blood antimony was determined at intervals after injection. No measurable amount could be detected after about 3 hr.

5. The antimony excreted during the first 24 hr. after injection of ter- or quinquevalent compounds was unchanged in valency. After an injection of Na antimony^{v} gluconate, part of the antimony remaining in the body was reduced to the tervalent state, but the quantity was small. Tervalent antimony was recovered from the livers of injected animals.

6. *In vitro* experiments have demonstrated the reduction of quinquevalent antimony by living tissues.

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The Biological Assay of Vitamin D_3

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Two methods are generally used for the biological assay of vitamin D_3 on chicks, viz. the A.O.A.C. method which uses the bone ash of the tibia as a criterion of response, and the method of Olsson [1936], in which the tarso-metatarsal distance (t.m.t.) measured on an X-ray film is so used. Both methods use criteria which are measured in fundamental units (mass or length, respectively). This gives them an advantage over those methods used in rat assays where a visual estimate is made by comparison with an arbitrary set of standards. The Olsson radiographic method also involves less time, expense and labour, since the chicks are not killed. The technique has been described elsewhere [Olsson, 1936; 1939; Baker & Wright, 1940] and the method has been adopted (1940) as standard by the British Standards Institute.

The present paper deals with some investigations on the nature of the test diet and its effect on the t.m.t. distance, and shows that on a suitable diet, gain in weight runs parallel with decrease in t.m.t. distance and affords a criterion for assay of vitamin D_3 of the same order of accuracy as the t.m.t. method. Experience with diet no. 1 of the British Standards Institute Specification 911/1940 indicated that differences in t.m.t. distances between

high and low doses of vitamin D were too small to be satisfactory, and resulted in a very low slope to the response curve. Baker & Wright [1940] quote cases where the difference between the t.m.t. distances for their extreme doses over an 8 : 1 range, is approximately 0·5 mm. Their experience is confirmed in this paper. Alternative diets were investigated with a view to increasing this difference. These diets comprised the A.O.A.C. diet used in the bone-ash test; a diet used by Olsson (private communication); and a diet used by Dr Wilkinson of Lever Bros. (private communication). Olsson's diet was a modification of his published diet [1936] in which bone meal is included.

METHODS

Chicks used. Sex-linked cockerels (Light Sussex and Rhode Island Red) were used in all experiments except in test 2, where pullets were used as well in equal number.

Housing. The chicks were housed in all-metal brooders, each of 6 units, arranged in three rows of two, each unit having a warm chamber, and an unheated run. The brooders were installed in a thermostatically controlled room with air circulation controlled by fans. Temperature in the heated chamber was 85–90° F. and in the room 80° F. during the first week, and it was lowered 5° F. each week for the next 4 weeks.

Food. Food was prepared in an electrically driven Hobart mixer. In all, six series of tests were made. Complete analyses of the diet were made in certain cases. Calcium and phosphorus determinations were done in all cases except series I. The composition and analysis of the diets are given in Tables 1 and 2. Variations between different batches of diet made to the same formula make it necessary to determine at least the Ca and P content of each batch, and to adjust these values to a constant figure, e.g. in diet A, suggested to us by Dr Wilkinson, values for CaO and P_2O_5 of 2·83 and 1·3–1·4% respectively were recommended, but the diet when made up for the test showed 1·09 and 2·24% respectively. The ratio was, therefore, adjusted by the addition of $CaCO_3$ so that the final quantities were 4·54% CaO and 2·24% P_2O_5. The results obtained with this diet differed from those obtained by Dr Wilkinson, possibly owing to the higher level of Ca and P, though the ratio is the same. There is evidence that doubling both Ca and P in Olsson's diet leads to smaller t.m.t. distances, but most of the chicks died within 6 weeks.

Table 1. *Composition of various diets fed to chicks*

Diets	B.S.I.	Diet A	A.O.A.C.	Olsson
Bran	16	15	—	—
Ground yellow corn	35	33·25	57	34·4
Middlings	23	38	28	28·4
Crude acid pptd. casein	—	—	12	—
Dried skim milk	8	—	—	9·4
Meat meal	10	—	—	—
Grass meal dried	5	—	—	5·5
Soya-bean meal	—	—	—	15·4
Bone meal	—	—	—	4·4
Fish meal	—	5	—	—
$Ca_3(PO_4)_2$	—	—	2	—
Iodized salt 0·02% KI	0·5	—	1	1·0
$MnSO_4.4H_2O$	—	0·0094	0·02	—
$CuSO_4$	—	0·0068	—	—
Charcoal	—	0·256	—	0·5
Ground limestone	1·5	4·0	—	—
NaCl	—	0·368	—	—
Fe_2O_3	—	0·111	—	—
Yeast	—	—	2	—
Dried cattle food yeast	—	2·5	—	—
Olive or arachis oil	1·0	1·0	1	1·0
Vitamin A oil (1000 i.u./g.)	—	0·5	—	—

Table 2

(*a*) *Analyses of various diets*

Material	% B.S.I.	A.O.A.C.	Olsson
Water	9·12	6·54	1·39
Fat	5·88	5·10	6·91
Protein (N × 6·25)	17·08	20·73	17·28
Starch	1·20	6·00	2·00
Sugar (reducing)	0·71	0·25	0·28
Ash	4·48	4·86	6·63
Ca	1·45	0·50	0·83
P	1·36	1·34	1·09
Fe	0·017	0·009	0·018
Mg	0·29	0·23	0·41

(*b*) *Ratio of calcium to phosphorus in various diets fed to chicks*

Diet		Test no. 2	3	4	5	6
B.S.I.:	Ca	1·45	1·37	0·84	—	—
	P	1·36	1·14	1·25	—	—
	Ratio	1·063	1·193	0·669	—	—
A.O.A.C.:	Ca	0·50	0·43	—	—	—
	P	1·34	0·91	—	—	—
	Ratio	0·358	0·450	—	—	—
Olsson:	Ca	—	—	0·83	0·94	1·17
	P	—	—	1·09	1·10	1·07
	Ratio	—	—	0·764	0·856	1·093

The Ca and P in the Olsson diet were maintained at 0·85–1·0 and 1·0–1·1% respectively, giving a ratio of Ca : P of approx. 1 : 1. In these separate experiments no adjustment was found necessary, the figures for Ca being 0·83, 0·94, 1·17, and for P 1·09, 1·10 and 1·07% respectively. The amount of food consumed by each group of birds was determined every week.

Vitamin D supply. The B.S.I. standard solution, i.e. crystalline vitamin D_3 dissolved in arachis oil to give a solution containing 1 unit/mg. of solution, was used, the units being calculated on the basis of 40×10^6/g. crystalline product. The solution was stored in a refrigerator and diluted with arachis oil to correspond to 1% of the ration, as required.

Procedure. 15–20 birds were allocated to a group (6–8 in negative controls). Day-old chicks were placed immediately on the basal diet without any addition of vitamin for 1 week. They were weighed and marked and again weighed at weekly intervals during the experiments. The dose of vitamin D_3 was incorporated in the diet from the 8th day onwards, and the birds were X-rayed when 4, 5 or 6 weeks old.

X-Ray technique. The tarso-metatarsal joints were X-rayed on Kodak Crystallex film with exposure of 1½ sec. at 12 in., 30 mA., 39 kV. Prints were made from the negatives on ultra-contrasting bromide paper, and the t.m.t. distances were measured, at a magnification of approx. 5,

with a travelling microscope (vernier) reading to 0·05 mm. An accuracy greater than this is not considered feasible. Whereas in general the edge of the tarsus was well defined even when magnified 5 times, the edge of the metatarsus was sometimes difficult to locate, and the best solution was found to be that of Halnan [Baker & Wright, 1940] which consists in marking with a scalpel a print viewed without magnification. Recent experiments show that the new Ilford Kryptoscreen X-ray paper gives good results and avoids the labour of printing. The reproducibility of such measurements is shown in Table 3A, which gives the data obtained from two sets of photographs, each of 20 bones, measured on each of 5 separate days, by two observers. The photographs were shuffled each time by a third person.

Table 3A. *Mean values of t.m.t. distances for sets of 20 photographs (mm.)*

	Set 1		Set 2	
	Observer A	Observer B	Observer A	Observer B
Reading 1	3·73 (5)	3·69 (8)	0·84 (3)	0·89 (5)
2	3·71 (3)	3·68 (0)	0·86 (0)	0·82 (5)
3	3·74 (5)	3·63 (0)	0·89 (0)	0·82 (0)
4	3·73 (0)	3·70 (0)	0·86 (8)	0·80 (3)
5	3·73 (8)	3·69 (5)	0·84 (5)	0·85 (0)
Overall mean	3·73 (2)	3·68 (1)	0·86 (1)	0·83 (9)
Mean difference between extreme readings for each bone	0·15 (5)	0·19 (7)	0·10 (5)	0·15 (0)

In a series of measurements of this same photograph therefore there is liable to be a difference between extreme readings of:

0·2 mm. in distances of approx. 4 mm.,
i.e. ±2·5% from the mean,

and of 0·15 mm. in distances of approx. 1 mm.,
i.e. ±7·5% from the mean.

In one experiment two sets of prints were made and marked and measured by two separate observers. The results are shown graphically in Fig. 1 and are summarized in Table 3B.

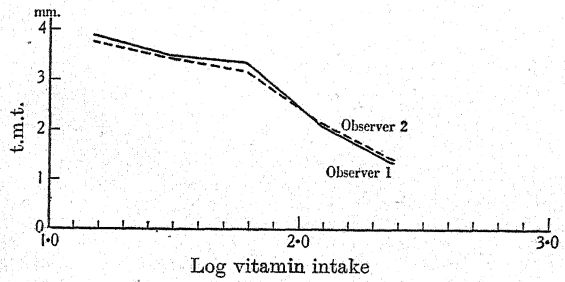

Fig. 1. Uniformity of t.m.t. measurements.

Table 3C shows assay results calculated from independent measurements made on separate sets of prints by two observers. The results calculated by the gain-in-weight method are included for comparison.

Table 3B. *Results of measurements by different observers on the same prints*

	Observer 1		Observer 2	
Dose group	Mean t.m.t.	Standard deviation	Mean t.m.t.	Standard deviation
5 u./100 g. diet	3·891	0·8112	3·771	0·7206
10 u./100 g. diet	3·739	0·8360	3·683	0·7856
15 u./100 g. diet	3·336	1·2020	3·178	1·1340
20 u./100 g. diet	2·131	0·8823	2·144	0·5825
40 u./100 g. diet	1·359	0·3871	1·382	0·5074
Weighted mean and pooled standard deviation	2·897	0·8151	2·838	0·7790

Table 3C. *Results of assays by different observers, t.m.t. method*

Material: C.L.O. emulsion reputed to contain 50 u./g.

	Observer 1		Observer 2			
Assay period weeks	Potency	Fiducial limits *P* 0·95 %	Potency	Fiducial limits *P* 0·95 %	Potency	Gain in wt. method %
4	47	76–126	52	79–123	48	75–131
5	46	78–124	46	78–124	46	79–125
6	48	75–129	—	—	47	79–123

RESULTS

Six experiments were made, but one, no. 3, was discarded because the chicks failed to grow. This was attributed by the hatchery to a breakdown of the incubators. The remaining experiments were as follows:

Exp. 1. An attempted assay using B.S.I. diet no. 1. It is considered unsatisfactory owing to the low slope of the regression line.

Exp. 2. Comparison of B.S.I. and A.O.A.C. diets. The results on the B.S.I. diet confirmed those in Exp. 1; those on the A.O.A.C. diet showed a greater slope of the regression line and a parallelism between t.m.t. distance and the reciprocal of the gain in weight.

Exp. 3. Discarded.

Exp. 4. Comparison of B.S.I., A & Olsson diets. B.S.I. and A diets showed similar results, which confirmed Exps. 1 and 2, viz. they gave relatively uniform growth at all dose levels, but very low slopes to the regression line. Olsson's diet gave results similar to the A.O.A.C. but appeared to be less severe in its effect at low doses. There was a linear relation (*a*) between gain in weight and vitamin D_3 intake, and (*b*) between t.m.t. and vitamin D_3 intake.

Exp. 5. Was confined to the Olsson diet. In addition to an assay of two oils, the effect of varying the Ca and P in the diets was examined. Doubling both Ca and P in the diet led to reduced t.m.t. distances and to reduced gains in weight, and many birds died. Doubling the P alone (sodium phosphate) led to death of all birds within 4 weeks. Doubling the Ca alone led to reduced t.m.t. distances and increased gain in weight.

Table 4. *B.S.I. Standard, t.m.t. data*

Test no.	Diet	Period of test, weeks	Dose levels, units/100 g. diet	t.m.t. distances	Slope	Pooled variance	Coefficient of pooled variation, %
1	B.S.I.	5	3–23	(1·767); 1·063; 0·88; 1·021; 0·885; 0·854	−0·1887	0·0932	32·45
		6	3–23	(1·325); 0·653; 0·618; 0·634; 0·485; 0·591	−0·1137	0·0689	40·04
2	B.S.I.	4	3–23	(0·819); 0·720; 0·889; 0·875; 0·961; 0·946	—	—	—
		5	3–23	(1·265); 0·947; 0·714; 0·760; 0·720; 0·700	−0·2212	0·0500	29·10
4	B.S.I.	4	2·5–20	(1·320); 1·361; 1·363; 1·650; 0·942	−0·5897	0·1820	36·15
		5	2·5–20	(1·280); [1·194]; 1·516; 0·932; 0·266	−2·2412	0·3686	67·35
2	A.O.A.C.	4	3–23	(3·757); 3·608; 3·353; 2·681; 2·436; 1·291	−2·0515	0·6264	29·43
		5	3–23	(2·769); 2·601; 2·368; 1·928; 1·604; 0·953	−1·4910	0·9074	49·57
4	A	4	2·5–40	(1·310); 1·420; 1·259; 1·132; 1·106; 1·222	−0·1826	0·1128	27·35
		5	2·5–40	(1·008); [1·547]; 2·059; 1·394; 1·165; 1·181	−0·9496	0·2666	35·70
4	Olsson	4	2·5–40	(3·450); [3·253]; 3·508; 2·494; 1·375; 0·808	−2·6184	0·5310	35·90
		5	2·5–40	(3·338); [3·500]; 3·767; 2·747; 0·761; [0·403]	−3·6800	0·8039	36·96
5	Olsson	4	5–40	(4·378); [3·590]; 2·950; 1·100; [0·788]	−4·8635	0·5983	38·20
		5	5–40	(3·925); 3·648; 2·700; 0·833; [0·759]	−3·7068	0·6981	34·91
		6	5–40	(3·372); 3·836; 2·898; 0·817; [0·691]	−3·7999	1·5408	49·00
6	Olsson	4	5, 10, 15, 20, 40	(3·643); [3·700]; 3·542; 2·768; 1·856; 0·765	−3·9777	0·6824	36·78
		5	,,	(4·000); [3·944]; 3·817; 3·091; 2·111; 0·682	−4·1662	0·6042	31·85
		6	,,	(3·760); [3·559]; 3·686; 3·123; 1·869; 0·912	−3·4988	0·8195	37·58

Table 5. *B.S.I. Standard. Gain-in-weight data*

Test no.	Diet	Period of test, weeks	Dose levels, u./100 g. diet	Gain in weight	Slope	Pooled variance	Coefficient of pooled variation, %
2	A.O.A.C.	4	3–23	(100·2); [94·89]; 83·56; 116·11; 158·28; 192·94	138·02	1103·4	24·28
		5	3–23	(166·75); [150·76]; 141·56; 188·00; 270·59	215·11	3059·6	24·21
4	Olsson	4	2·5–40	(138·8); [121·4]; 124·3; 204·5; 257·2; [258·4]	178·75	2685·5	26·36
		5	2·5–40	(182·5); [147·8]; 150·3; 277·7; 351·2; [370·6]	255·79	4563·2	25·63
5	Olsson	4	5–40	(119·05); 143·65; 175·75; 273·90; 322·71	182·19	2193·1	20·8
		5	5–40	(142·82); [169·50]; 239·35; 413·35; [475·4]	418·17	4577·0	20·70
		6	5–40	(162·55); 192·61; 281·20; 524·65; [583·82]	433·61	7369·5	25·43
6	Olsson	4	5, 10, 15, 20, 40	(101·5); 125·1; 162·6; 190·2; 212·7; 251·2	119·74	1416·6	19·87
		5	,,	(123·2); 165·0; 201·5; 256·9; 312·2; 397·0	212·57	3486·6	22·17
		6	,,	(139·6); [185·0]; 234·0; 310·1; 400·6; 539·1	373·19	7494·8	23·43

Table 6. *Assays by t.m.t. method*

Test no.	Diet	Period of test, weeks	Material	Dose levels: Standard	Dose levels: Sample mg.	Combined slope	Pooled variance	Potency	Fiducial limits $P=0·95$ %
2	A.O.A.C.	4	C.L.O.	3–23	25 and 76·4	−1·9588	0·6072	112	64–151
5	Olsson	4	C.L.O.	10–20	100 and 200	−5·1106	0·5973	65	82–118
		5	,,	10–20		−4·7128	0·8074	62	78–125
		6	,,	10–20		−4·5866	0·8492	86	80–123
5	Olsson	4	Veterinary C.L.O. blended with seal oil	10–20	500 and 1000	−4·9687	0·6636	17	85–119
		5	,,	10–20		−4·3095	0·7756	16	80–123
		6	,,	10–20		−4·5724	0·8084	18	81–124
6	Olsson	4	C.L.O. emulsion	5, 10, 15, 20, 40	200, 300, 400	−3·7933	0·8081	47	76–126
		5	,,	,,	,,	−3·8322	0·9620	46	75–124
		6	,,	,,	,,	−3·4700	1·1161	48	75–129

Table 7. *Assays. Gain in weight*

Test no.	Diet	Period of test, weeks	Material	Dose levels: Standard	Dose levels: Sample mg.	Combined slope	Pooled variance	Potency	Fiducial limits $P=0{\cdot}95$ %
2	A.O.A.C.	4	C.L.O.	3–23	25 and 76·4	119·75	925·9	143	74–131
5	Olsson	4	C.L.O.	16–20	100 and 200	230·23	2163·2	71	77–124
		5	,,	—	—	400·45	4232·3	80	85–120
		6	,,	—	—	488·03	11710·4	74	77–126
5		4	Veterinary C.L.O. blended with seal oil	16–20	500 and 1000	214·08	2047·1	21	81–128
		5	,,	—	—	377·05	4211·9	21	84–121
		6	,,	—	—	498·90	9869·9	20	81–124
6	Olsson	4	C.L.O. emulsion	5, 10, 15, 20, 40	200, 300, 400	119·20	1293·6	47	75–131
		5	,,	,,	,,	210·95	3587·3	46	79–125
		6	,,	,,	,,	362·31	8114·7	47	79–123

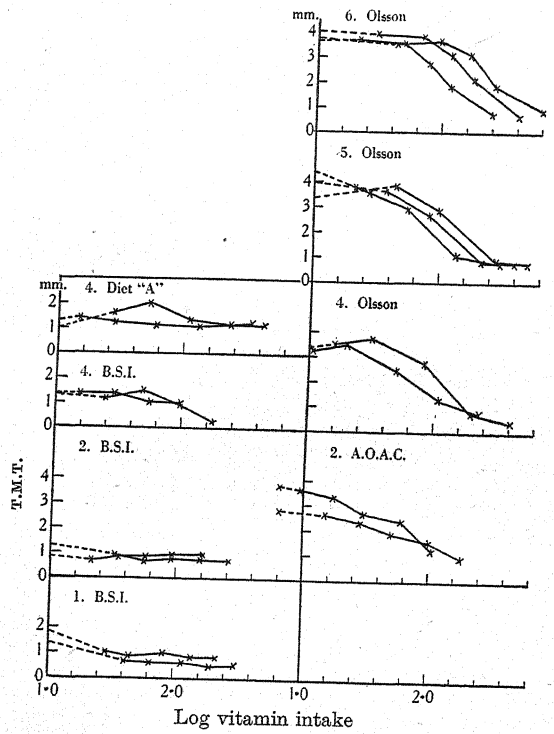

Fig. 2. T.M.T. responses.

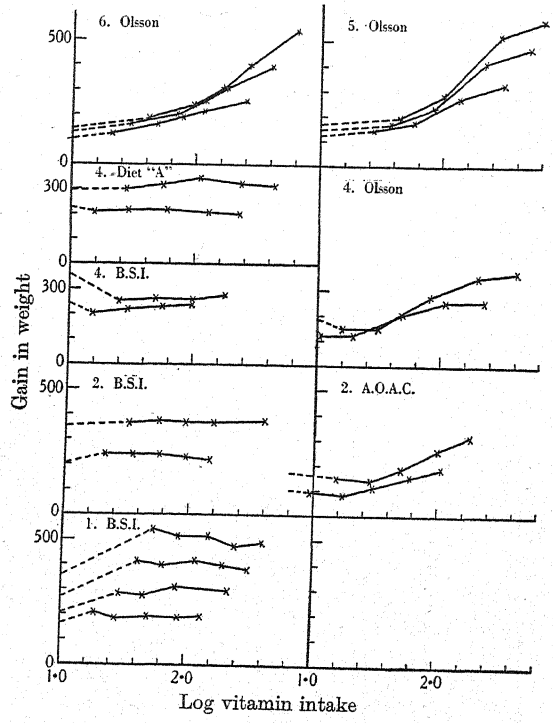

Fig. 3. Gain in weight responses.

Exp. 6. Was also confined to the Olsson diet in an assay. A parallel set of standards was run in which the vitamin was separately administered to each chick by micrometer syringe. This mode of administration showed slightly greater weight increases for the same vitamin intake, but paradoxically, with the higher doses, greater t.m.t. distances. This point is being further investigated. The observations of Fritz, Halpin, Hooper & Kramke [1942] on oxidative destruction of vitamin D in poultry foods makes it important to investigate these two methods of dosing for assay purposes.

The essential results of the experiments are given in Tables 4–7 and in Figs. 2 and 3. Tables 4 and 5 are concerned only with responses to the B.S.I. standard vitamin D_3 preparation; Tables 6 and 7 give certain assay results. Dose levels were (in addition to negative controls) 3, 5, 8·33, 13·68 and 23 units/100 g. diet or 2·5, 5, 10, 20 and 40 units/100 g. diet. In some cases, as will be obvious from the figures, extreme doses had to be omitted to satisfy criteria of linearity, and these values are enclosed in square brackets. Negative control values are shown in curved brackets. There is

general agreement that the linear response occurs with doses from 10 to 25 units/100 g. diet. Above 25 units no great change occurs, and below 10 there is little calcification. This is confirmed in test 6, which includes a dose level of 15 units/100 g. diet.

One of the advantages claimed for the B.S.I. diet is that growth is equalized and is independent of vitamin intake. Olsson's data show that over the active dose range 8–32 units/100 g. food (he shows no point between 16 and 32 units), there is a gain in weight of about 40–50 g. after 4 weeks. This is about one-third of the gain in our experiments, and Olsson introduces a correction for his t.m.t. values to compensate for the differences in weight of the chicks. Where, however, as in our results, the gain and t.m.t. are related, such a correction is not necessary. A probable reason for the difference between our results and Olsson's lies in the Ca and P content of the diet. Olsson gives 1·76% Ca and 1·04% P, against our figures of 1·0 and 1·1% respectively.

Table 8. *Assay by log t.m.t. method*

Test no.	Diet	Period of test, weeks	Material	Combined slope	Pooled variance	Potency	Fiducial limits $P=0{\cdot}95$ %
5	Olsson	4	C.L.O.	−1·0384	0·03615	61	78–123
		5	„	−1·0090	0·03196	57	78–122
5	Olsson	4	Veterinary C.L.O. blended with seal oil	−1·2171	0·04182	17	84–119
		5	„	−1·099	0·037973	16	82–120

DISCUSSION

It will be seen that the slope of the regression line for the B.S.I. diet is very low, and that there is a tendency to greater regularity when the test period is longer than 4 weeks. In test 2, using this diet, the t.m.t. after 4 weeks on the highest doses is greater than that on the lowest doses, but the difference is not statistically significant. After 5 weeks, though there is a reduction in t.m.t. with increasing dose, the difference between responses for extreme groups is not statistically significant.

In marking the end of the metatarsus for measurement, there are occasions when the end of the bone is so ill defined that a difference of 0·5 mm. may occur in marking the same photograph on different occasions. The greater the t.m.t. distance, the less the relative magnitude of such an error, and since such photographs occur mostly on low doses, it is highly desirable that the t.m.t. distances should be great on such doses so as to minimize this error.

On the A.O.A.C. diet the slope of the regression line is much increased, but after 5 weeks it is less so than after 4 weeks. The Olsson diet gives good slopes, which, as shown in the diagrams for tests 4–6, are consistent and not affected by the duration of the test between 4 and 6 weeks.

In these tests, the t.m.t. distance has been used rather than its logarithm, as recommended in the B.S.I. specification. Assays in this series calculated by both methods (t.m.t. and log t.m.t.) gave almost identical results for potency and fiducial limits (Tables 6 and 8). The object of using the log t.m.t. is presumably to satisfy the mathematical purist's desire to equalize the variance in the different groups. It may be mentioned here that this is not necessary when the gain in weight is used as the criterion of response (see below).

On the Olsson diet, as used in these experiments, there is a marked difference in growth (and a corresponding difference in food intake) with increasing vitamin D dose level; this makes it necessary to use intake instead of dose level in calculating the results. It will be seen from the figures that this increase is linearly proportional to the log of the intake, and this being so, there is no need to correct the t.m.t. distances for growth differences. On the contrary, the weight increase is in itself a satisfactory criterion of response, and has the advantage of giving an equalized variance between groups, and also a lower coefficient of pooled variation. In some assays it tends to give higher results, which however, are more consistent over different test periods than the t.m.t. results and are of a similar degree of reliability. The slope of the response curve increases when the test is prolonged and we favour a 5 weeks' test period when gain in weight is used as a criterion.

SUMMARY

In the light of the results described above, it is suggested that the method of using the growth response of chicks is a simple, convenient and accurate method for assays of vitamin D_3, when the rachitogenic diet is suitably chosen and controlled, with special regard to its Ca and P content.

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The *in vitro* Oxidation of Pyruvic and α-Ketobutyric Acids by Ground Preparations of Pigeon Brain. The Effect of Inorganic Phosphate and Adenine Nucleotide

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Quantitatively, pyruvate oxidation in pigeon brain has mainly been investigated with minced tissue [Peters & Thompson, 1934; McGowan, 1937; Long, 1938]. While most of the pyruvate is oxidized completely to CO_2 and water, small amounts undergo either oxidative decarboxylation (reaction 1; $R = H$) or anaerobic dismutation (reaction 2; $R = H$).

$$2R\mathrm{CH_2.CO.COOH} + \mathrm{O_2} \rightarrow 2R\mathrm{CH_2.COOH} + 2\mathrm{CO_2}, \quad (1)$$

$$2R\mathrm{CH_2.CO.COOH} + \mathrm{H_2O} \rightarrow R\mathrm{CH_2.COOH} + R\mathrm{CH_2.CH(OH).COOH} + \mathrm{CO_2}. \quad (2)$$

α-Ketobutyric acid is not a normal metabolite in biological systems, nor does the corresponding *l*-α-amino-butyric acid seem to occur naturally [Dunn, 1938], so that the activation of this keto-acid by enzymes must be regarded as an artefact. Nevertheless, its behaviour towards minced pigeon brain has led to valuable conclusions concerning the mechanism of pyruvic acid utilization [McGowan & Peters, 1937; Long & Peters, 1939; Long, 1942].

Minced pigeon brain could not completely oxidize α-ketobutyric acid, although oxidative decarboxylation (reaction 1; $R = CH_3$) and anaerobic dismutation (reaction 2; $R = CH_3$) occurred readily [Long & Peters, 1939]. Nevertheless, pyruvic and α-keto-butyric acids were equally rapidly utilized [Long, 1942], that fraction which was completely oxidized in the case of pyruvic acid merely undergoing oxidative decarboxylation in the case of α-keto-butyric acid.

Banga, Ochoa & Peters [1939 *a*, *b*], using dialysed pigeon brain suspensions in which the cell structure of the tissue was destroyed by grinding, found that pyruvate oxidation was stimulated by the addition of inorganic phosphate, adenine nucleotide and fumarate. Ochoa [1939] showed that Mg^{++} was also required. This enzyme system seems to have little in common with the pyruvate oxidation system as it occurs in the minced tissue, the respiration of which is not affected by the addition of these dialysable components; but evidence will be presented to show that this is not due to the presence in the minced brain of these activators.

In the present work enzyme studies with ground preparations of pigeon brain have been used to assess the effects of added inorganic phosphate and adenine nucleotide on the oxidation of both pyruvic and α-ketobutyric acids: a further communication will deal with fumarate catalysis. The oxidation of both pyruvate and α-ketobutyrate has been found to be stimulated by inorganic phosphate. That of pyruvate was also markedly increased by the addition of adenine nucleotide, but the latter's effect on α-ketobutyrate oxidation was only very slight. The increased O_2 uptake, pyruvate utilization and O_2/pyruvate ratio, together with the lowered R.Q., which were observed in presence of adenine nucleotide, showed that pyruvate was much more completely oxidized in the presence of the nucleotide than in its absence.

EXPERIMENTAL

Materials

Pyruvic and α-ketobutyric acids [Peters, 1938; Long & Peters, 1939]. The pure crystalline Na salts were used. Final concentration $M/100$, unless otherwise stated.

Adenine nucleotide. Three preparations were employed: (1) muscle adenylic acid (a gift from Prof. R. A. Peters), (2) adenylpyrophosphoric acid (Henning), and (3) Ca acid adenylpyrophosphate (a gift from Prof. T. H. Milroy). This last specimen was freed from Ca by Na oxalate. All three preparations, after neutralization, had the same effect on the oxidation of pyruvate by dialysed suspensions of pigeon brain containing $M/20$ phosphate buffer, *p*H 7·3. Adenine nucleotide was used throughout in concentration producing the maximum effect (0·1 mg./ml. $= 3 \times 10^{-4} M$).

Methods

(1) *The estimation of pyruvic and α-ketobutyric acids*

This was by the method of Lu [1939] as modified by Long [1942].

(2) *The estimation of inorganic phosphate*

The method of Berenblum & Chain [1938] was applied in the following way to small volumes (2 ml.) of protein-free filtrates containing very low concentrations ($0{\cdot}5$–$2{\cdot}5 \times 10^{-4} M$) of inorganic phosphate.

Into three test tubes (*a*), (*b*) and (*c*) were measured: (*a*) 2 ml. of the 5% trichloroacetic acid, protein-free filtrate,

(*b*) 2 ml. of the standard phosphate ($2 \times 10^{-4} M$) in 5% $CCl_3.COOH$, and (*c*) 2 ml. 5% $CCl_3.COOH$ (control). 10*N* H_2SO_4 (0·1 ml.), 5% ammonium molybdate (1 ml.) and *iso*butanol (4 ml.) were added to each. The contents were rapidly drawn into and expelled from a teat-pipette about 30 times. The lower aqueous layers were removed, by means of the corresponding pipettes, and discarded. The *iso*butanol extracts were then washed by extracting twice with 2 ml. *N* H_2SO_4. 0·4% $SnCl_2$ in *N* H_2SO_4 (3 ml.), freshly prepared from 40% $SnCl_2$ in conc. HCl, was then added to each. After thorough mixing and removal of the aqueous layer, each extract was made up to 10 ml. with ethanol. The absorption intensities were compared in a Hilger Spekker absorptiometer, using an orange filter (Hilger no. 3). The concentration of inorganic phosphate is linearly related to the absorption.

(3) *The preparation of the enzyme suspensions*

(*a*) *For anaerobic experiments.* Exhaustive washing of the tissue was necessary in order to produce a very low residual activity. The method of preparation [cf. Long & Peters, 1939] was as follows:

The cerebrum and optic lobes (about 1·5 g.) were removed as rapidly as possible from a freshly killed pigeon, and ground, ice-cold, in a mortar for 10 min. 0·9% KCl (6 ml.), ice-cold, was then added in portions and the grinding continued for a further 5 min. After passing through muslin, the brain suspension was centrifuged and the supernatant fluid discarded. The deposit was washed twice with 0·9% KCl and then three times with 0·2% KCl (6 ml. each time), keeping throughout as cold as possible and allowing the salt solutions to remain in contact with the enzyme for about 15 min. before each centrifugation. The washed, almost colourless, suspension, now free from cytochromes, was made up to 10 ml. with 0·2% KCl. 1 ml. suspension was equivalent to 150 mg. fresh brain tissue.

(*b*) *For aerobic experiments.* The preparation of dialysed brain suspensions, except in the temperature during extraction, resembled that of the 'dispersions' described by Banga *et al.* [1939*b*].

The cerebrum and optic lobes (about 4·5 g.) from three pigeons were placed in an ice-cold mortar and ground for 10 min. The contents warmed to room temperature during grinding. 0·9% KCl (9 ml.), ice-cold, was then added in portions and the grinding continued for 5 min. The brain suspension was passed through muslin, pipetted into two freshly prepared collodion sacs and dialysed for 3 hr. (unless otherwise stated) against 0·4% KCl (3 l.) at below 2°. During dialysis, the sacs were mechanically agitated. 1 ml. suspension was equivalent to 333 mg. fresh brain tissue.

(4) *Thunberg technique*

The Thunberg tubes [Keilin, 1929] contained 1 ml. of the enzyme suspension (added last), and substrates and other constituents to make the total volume 1·8 ml., the *p*H of the mixture being 7·3. The hollow stopper contained 0·2 ml. methylene blue (1 : 5000). The tubes were twice exhausted and filled with N_2 and again exhausted. At zero time the methylene blue was added to the other constituents; temperature 38°. Duplicate determinations were always made. 100 × the reciprocal of the reduction time (*t* min.) was taken as a measure of the activity of the system.

(5) *Respiration technique*

1 ml. of the enzyme suspension, prepared as under (3) (*b*), was shaken at 28° in air in a total volume of 2 ml. at *p*H 7·3, usually for 40 min. O_2 uptakes were determined in Barcroft flasks, with filter paper soaked in 2*N* KOH (0·2 ml.) in the centre well. O_2 uptake was determined in duplicate or triplicate, and the agreement was ±1·5%. If O_2 uptakes were not required, the suspension was shaken in 50 ml. Erlenmeyer flasks in the thermostat at 28°. For α-keto-acid or phosphate estimations, 25% $CCl_3.COOH$ (1 ml.) was added to the medium at the beginning and end of the respiration period. It was allowed to stand 15 min., the protein precipitate was filtered off, and the flasks and filters rinsed with 5% $CCl_3.COOH$ (3 × 2 ml.). After standing overnight, the volumes were made up to 10 ml. and aliquots taken for α-keto-acid or phosphate estimation.

Results

(1) *The effect of inorganic phosphate on the oxidation of pyruvic and α-ketobutyric acids by ground preparations of pigeon brain*

Lipmann [1937] has shown that inorganic phosphate or arsenate is necessary for O_2 uptake with acetone-dried preparations of *B. delbrükii* in the presence of pyruvate and methylene blue. The relation of O_2 absorbed and CO_2 produced to pyruvate utilized, indicated oxidative decarboxylation to acetic acid. Banga *et al.* [1939*b*] observed that phosphate markedly increased the O_2 uptake of dialysed pigeon brain suspensions in presence of pyruvate, adenine nucleotide, fumarate and Mg^{++}. They inferred that the respiration would be zero in the complete absence of inorganic phosphate. To simplify the problem, the experiments described in this section were carried out in order to test the effect of inorganic phosphate, only, on the oxidation of pyruvate and α-ketobutyrate by pigeon brain suspensions, in the absence of other factors.

Anaerobic experiments. The time taken by the system, dehydrogenase preparation and α-keto-acid, to reduce 0·2 ml. methylene blue (1 : 5000) was determined by the Thunberg technique in the presence and absence of phosphate buffer, *M*/20, *p*H 7·3. The osmotic pressure and Na^+/K^+ ratio of the media were kept constant throughout, isotonic phosphate and chloride being interchanged.

There is no doubt that inorganic phosphate exerts a powerful catalytic effect on the anaerobic oxidation of both pyruvate and α-ketobutyrate (Tables 1 and 2). The increased activity due to phosphate addition is quantitatively the same for the two α-keto-acids.

Two typical curves connecting phosphate concentration and activity are shown in Fig. 1, in which the effect of phosphate in the absence of α-keto-acid is also included in one case. The residual concentration of phosphate in the tissue was found to be $0{\cdot}75 \times 10^{-4} M$. Very small concentrations of inorganic phosphate exert a pronounced catalytic

Table 1. *The anaerobic oxidation of pyruvate and α-ketobutyrate in the presence and absence of inorganic phosphate*

	Activity (100/t)					
	Exp. 1			Exp. 2		
Substrate	Control	Phosphate	Diff.	Control	Phosphate	Diff.
Nil	0·5	0·5	0·0	1·1	2·5	1·4
Pyruvate	0·5	15·4	14·9	5·3	14·8	9·5
α-Ketobutyrate	0·5	14·8	14·3	4·0	13·8	9·8

t = reduction time (min.).

effect on the oxidation, and extrapolation to zero phosphate concentration shows that some α-keto-acid oxidation still occurs.

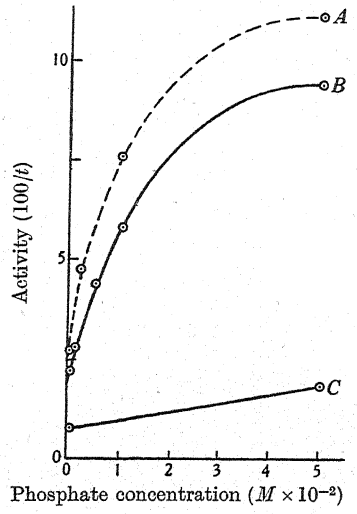

Fig. 1. The effect of inorganic phosphate concentration on the activity of the α-keto-acid dehydrogenase system from pigeon brain. Curve *A*, pyruvate; curve *B*, α-ketobutyrate; curve *C*, no substrate. Curves *B* and *C* refer to the same experiment, curve *A* to a separate experiment.

Arsenate also exerts a definite effect on the anaerobic oxidation of both pyruvate and α-keto-butyrate, and this effect is quantitatively the same for the two α-keto-acids (Table 2). At equivalent concentration ($M/20$), arsenate appears to be about 70–75 % as active as phosphate.

Table 2. *The replacement of phosphate by arsenate in the anaerobic oxidation of pyruvate and α-keto-butyrate*

		Activity (100/t)				
Exp.	Substrate	Control	Phosphate	Diff.	Arsenate	Diff.
1	Nil	0·5	0·7	0·2	0·7	0·2
	Pyruvate	0·9	6·9	6·0	5·7	4·8
	α-Ketobutyrate	0·5	6·5	6·0	5·3	4·8
2	Nil	0·5	0·9	0·4	0·9	0·4
	Pyruvate	2·1	10·0	7·9	7·5	5·4
	α-Ketobutyrate	0·9	9·3	8·4	7·1	6·2

t = reduction time (min.).

Aerobic experiments. Table 3 shows that the net O_2 uptake due to oxidation of pyruvate or α-keto-butyrate by brain suspensions is approximately doubled on addition of $M/20$ phosphate. The osmotic pressure and Na^+/K^+ ratio of the media were again kept constant throughout.

The catalytic effect of phosphate shown in Table 3 does not appear to be as great as that obtained under anaerobic conditions. The residual inorganic phosphate concentration of the tissue, however, is very different in the two cases. The dehydrogenase preparation contained only about $0{\cdot}75 \times 10^{-4} M$, whereas the dialysed tissue suspension contained about $1{\cdot}1 \times 10^{-3} M$ phosphate. The comparison between the aerobic and anaerobic oxidations should therefore be made at two definite phosphate concentrations, e.g. $1{\cdot}1 \times 10^{-3} M$ and $5 \times 10^{-2} M$. Thus in one anaerobic experiment with α-ketobutyrate as substrate, the activity at $1{\cdot}1 \times 10^{-3} M$ was 3·5, increasing to 7·4 at $5 \times 10^{-2} M$ phosphate; increase about 114 %. This figure agrees quite closely with the percentage increase found for the catalytic effect in respiring pigeon brain suspensions.

In contrast to its stimulating action on the anaerobic oxidation of α-keto-acids, arsenate did not cause an increased respiratory rate when tested with the dialysed brain suspension in the presence of pyruvate or α-ketobutyrate (Table 4). Not only is the net O_2 uptake due to α-keto-acid oxidation

Table 3. *The effect of inorganic phosphate on the respiration of dialysed pigeon brain suspensions in the presence of pyruvate and α-ketobutyrate*

		O_2 uptake (μl./40 min.)						
		No added phosphate			Added phosphate ($M/20$)			
Substrate	Exp.	Control	With substrate	Net	Control	With substrate	Net	Net increase %
Pyruvate	1	52·4	100·5	48·1	51·0	149·9	98·9	103
α-Ketobutyrate	2	51·8	79·5	17·7	60·7	117·0	56·3	103
	3	54·1	75·3	21·2	65·4	108·0	42·6	101
	4	52·4	62·5	10·1	51·0	75·0	24·0	138

decreased by arsenate addition, but so also is the residual respiration rate. The cause of this generalized inhibition has not been investigated.

Table 4. *The effect of arsenate on the respiration of dialysed pigeon brain suspensions in the presence of pyruvate and α-ketobutyrate*

		O_2 uptake (μl./40 min.)					
		No arsenate added			Arsenate added ($M/40$)		
Substrate	Exp.	Control	With substrate	Net	Control	With substrate	Net
Pyruvate	1	52·4	100·5	48·1	43·4	80·6	37·2
α-Ketobutyrate	2	49·4	76·1	26·7	38·4	50·5	12·1
	3	52·4	62·5	10·1	43·4	50·3	6·9

The experiments recorded in Table 5 show that although the addition of phosphate to brain suspensions brings about an increased utilization of α-keto-acid in all cases, this increase, which does not exceed 50 %, is perhaps less than might have been expected. The effect of various phosphate concentrations on the utilization of α-keto-acid is shown in Fig. 2 (two typical experiments, using

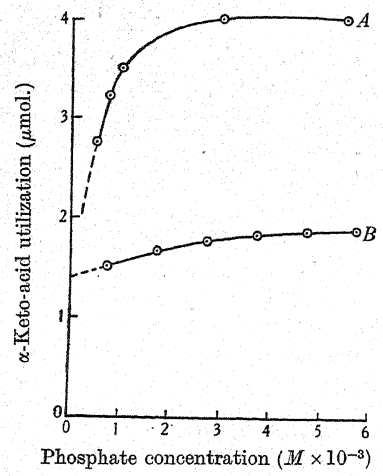

Fig. 2. The effect of inorganic phosphate concentration on the utilization of α-keto-acids by respiring dialysed pigeon brain suspensions. Curve *A*, pyruvate; curve *B*, α-ketobutyrate. The two curves refer to separate experiments.

pyruvate and α-ketobutyrate as substrates). Extrapolation of the curves to zero phosphate concentration shows that even in the complete absence of inorganic phosphate there may be an appreciable utilization of α-ketobutyrate. It is difficult to draw any definite conclusion regarding the utilization of pyruvate at zero phosphate concentration, since the curve is so steep at the point of extrapolation.

Table 5. *The utilization of pyruvate and α-ketobutyrate in the presence and absence of added inorganic phosphate*

			Added phosphate conc.	α-Keto-acid utilization μmol.		
Substrate	Exp.	Dialysis hr.	$M \times 10^{-3}$	Nil	Phosphate	Increase %
Pyruvate	1	3	50	1·99	3·08	55
	2	3	10	2·92	4·31	47
	3	3	10	2·80	4·01	50
	4	3	5	1·55	1·80	16
	5	24	1·2	0·69	0·83	22
α-Ketobutyrate	6	3	5	1·96	2·14	9
	7	7	5	1·51	1·89	25

(2) *The comparative utilization of pyruvic and α-ketobutyric acids by washed preparations of minced pigeon brain and dialysed pigeon brain suspensions in the presence of phosphate*

The quantitative comparison of keto-acid utilization by these two systems has not previously been made. The results have been expressed in terms of μmol. of α-keto-acid utilized in 40 min. by an amount of enzyme preparation corresponding to 1 g. fresh tissue. The washed mince was found to contain an average of 87·8 % water, as compared with 79·8 % in the fresh brain tissue. Taking this factor into account, the average values found in a number of experiments for α-keto-acid utilization are quoted in Table 6. The figures indicate (1) that pyruvic and α-ketobutyric acids are equally rapidly utilized by washed pigeon brain mince [cf. Long, 1942], (2) that the utilization of α-ketobutyric acid is the same in washed mince and dialysed suspensions, and (3) that pyruvic acid is more rapidly utilized by dialysed suspensions than by washed mince. This last point will be referred to later.

Table 6. *The utilization of pyruvate and α-ketobutyrate by washed pigeon brain mince and dialysed pigeon brain suspensions in the presence of phosphate*

Phosphate concentration—$M/20$ in suspensions, $M/10$ in minced brain. Respiration at 28° in air, for 40 min.

	Pyruvic acid		α-Ketobutyric acid	
	Washed mince	Dialysed suspension	Washed mince	Dialysed suspension
No. of determinations	3	14	5	13
α-Keto-acid utilization, μmol./g. tissue/40 min.	10·4	13·5	9·6	9·6
Standard deviation of mean (ϵ)	0·3	0·9	0·8	0·8

(3) *The effect of adenine nucleotide on the oxidation of pyruvic and α-ketobutyric acids by ground preparations of pigeon brain*

Banga *et al.* [1939*b*] observed that the omission of adenine nucleotide from the system (dialysed

pigeon brain suspension, pyruvate, inorganic phosphate, adenine nucleotide, fumarate and Mg^{++}) much reduced the rapid respiration rate. The following series of experiments was undertaken to discover what was the action of adenine nucleotide on the simplified system, dialysed brain suspension, pyruvate and inorganic phosphate only. In some experiments pyruvate was replaced by α-ketobutyrate.

The anaerobic oxidation of pyruvic and α-ketobutyric acids in the presence and absence of adenine nucleotide. Adenine nucleotide was found to be without effect on the anaerobic oxidation of either pyruvate or α-ketobutyrate (Thunberg technique; Table 7).

Table 7. *The anaerobic oxidation of pyruvate and α-ketobutyrate in the presence and absence of adenine nucleotide*

M/20 phosphate present throughout

	Activity (100/*t*)					
	Exp. 1			Exp. 2		
Substrate	Nil	Adenine nucleotide	Diff.	Nil	Adenine nucleotide	Diff.
Nil	0·5	0·5	0·0	1·0	1·0	0·0
Pyruvate	15·4	12·5	−2·9	11·1	11·1	0·0
	Exp. 3			Exp. 4		
Nil	0·9	1·1	0·2	0·7	0·8	0·1
Pyruvate	11·8	12·9	1·1	—	—	—
α-Ketobutyrate	—	—	—	10·5	11·4	0·9

t = reduction time (min.).

Table 8. *The effect of adenine nucleotide on the respiration of dialysed pigeon brain suspensions in the presence of pyruvate and α-ketobutyrate*

Suspension dialysed for 3 hr. Respiration at 28° in air for 40 min. *M*/20 phosphate present throughout. 1 ml. suspension = 333 mg. fresh brain tissue.

		O_2 uptake (μl.)					
		No addition			Adenine nucleotide		
Substrate	Exp.	Nil	With substrate	Net	Nil	With substrate	Net
Pyruvate	1	56·2	135·2	79·0	64·7	291·0	226·3
	2	105·0	196·7	91·7	140·3	370·0	229·7
	3	71·0	119·7	48·7	95·3	272·0	176·7
	4	73·7	130·6	56·9	110·0	325·0	215·0
	5	64·1	151·2	87·1	76·3	271·0	194·7
	6	38·1	93·5	55·4	49·5	235·5	186·0
	7	88·6	159·2	70·6	118·1	305·0	186·9
	8	70·5	126·5	56·0	102·2	265·5	163·3
	9	61·0	132·2	71·2	84·2	319·0	234·8
	10	58·7	141·8	83·1	70·1	284·5	215·4
	Av.	68·7	138·7	70·0	91·1	293·9	202·8
α-Keto-butyrate	11	74·3	142·4	68·1	88·5	154·2	65·7
	12	71·0	126·0	55·0	74·2	133·8	59·6
	13	83·7	130·7	47·0	89·1	139·4	50·3
	14	71·0	113·3	42·3	95·3	151·8	56·5
	15	43·6	70·9	27·3	49·0	95·0	46·0
	Av.	68·7	116·7	48·0	79·2	134·8	55·6

The effect of adenine nucleotide on the O_2 uptake of dialysed pigeon brain suspensions in the presence of pyruvic and α-ketobutyric acids. The suspensions contained added inorganic phosphate. With pyruvate, the average net O_2 uptake due to oxidation of this substrate, in the absence of added adenine nucleotide, was about 70 μl./333 mg. tissue/40 min. (Table 8). The value was almost trebled when adenine nucleotide was added (cf. columns 4 and 7). With α-ketobutyrate as substrate, on the other hand, adenine nucleotide seemed to have little effect on the net O_2 uptake, although in two cases (Exps. 14 and 15) a definite increase was observed.

It should be noted that in order to observe any catalytic effect of adenine nucleotide on pyruvate oxidation by dialysed pigeon brain suspensions, inorganic phosphate must always be included in the respiration medium. In the experiment quoted below the effect of phosphate was studied on the respiration of dialysed pigeon brain suspension with $3 \times 10^{-4} M$ adenine nucleotide present throughout.

O_2 uptake (μl.)					
No phosphate added			*M*/20 phosphate added		
Nil	Pyruvate	Net	Nil	Pyruvate	Net
17·0	35·9	18·9	58·6	209·5	150·9

In the absence of phosphate, the net O_2 uptake due to pyruvate oxidation is of the same order as that obtained under comparable experimental conditions in the absence of both phosphate and adenine nucleotide (Tables 3 and 4). In the presence of phosphate, however, the net O_2 uptake is increased about eightfold.

The effect of adenine nucleotide on the utilization of pyruvic and α-ketobutyric acids by dialysed pigeon brain suspensions. The utilization of pyruvate is approximately doubled when adenine nucleotide is added (Table 9). Under similar conditions (Table 9,

Table 9. *The effect of adenine nucleotide on the utilization of pyruvate and α-ketobutyrate by dialysed pigeon brain suspensions*

			α-Keto-acid utilization μmol.	
	Exp.	Dialysis hr.	No addition	Adenine nucleotide
Pyruvate	1	3	4·74	10·05
	2	3	5·81	10·27
	3	3	4·53	9·90
	4	3	3·07	7·83
	5	3	5·32	9·89
	6	3	4·20	10·18
	7	3	4·27	9·24
	8	3	4·04	7·99
		Av.	4·50	9·43
α-Ketobutyrate	9	3	4·57	4·60
	10	3	3·17	4·27
		Av.	3·87	4·43
	11	8	1·75	2·43
	12	8	2·13	3·35
		Av.	1·94	2·89

Exps. 9 and 10), there exists no corresponding effect with α-ketobutyrate as substrate. When, however, the brain suspension is dialysed for longer periods, the effect of adenine nucleotide becomes more evident (Table 9, Exps. 11 and 12). This observation, taken in conjunction with the occasional effect of adenine nucleotide on the O_2 uptake of brain suspensions respiring in α-ketobutyrate (Table 8, Exps. 14 and 15), indicates that the nucleotide does play a definite part in α-ketobutyrate oxidation. It appears that the α-ketobutyrate oxidation system is saturated by minimal concentrations of adenine nucleotide, so that after a period of dialysis lasting only 3 hr., sufficient usually remains associated with the enzyme to produce maximal effects.

(4) *The molecular ratios of net O_2 absorbed to α-keto-acid utilized for pyruvic and α-ketobutyric acid oxidation in respiring pigeon brain suspensions*

McGowan [1937] found for washed preparations of minced pigeon brain that 1·77 μmol. O_2 were absorbed per μmol. pyruvic acid utilized. From R.Q. data [Long & Peters, 1939] the corresponding molecular ratio for α-ketobutyric acid may be calculated. It lies between 0·37 and 0·45. This ratio has now been determined experimentally by estimation of α-ketobutyric acid [Long, 1942], and for comparison with respiring suspensions of pigeon brain; the values obtained are included in Table 10.

Table 10. *The molecular ratio of net O_2 absorbed to α-ketobutyric acid utilized by washed preparations of minced pigeon brain*

Washed minced pigeon brain prepared according to Long & Peters [1939]. Initial concentration of α-ketobutyrate, *M*/50.

Exp.	Temp. of respiration °C.	O_2 uptake (μmol./g./hr.) Control	α-Ketobutyrate	Net	α-Ketobutyric acid utilization (μmol./g./hr.)	O_2/α-ketobutyrate (molecular ratio)
1	28	6·92	10·18	3·26	9·75	0·33
2	28	6·63	9·16	2·53	6·14	0·41
3	28	7·01	10·38	3·37	8·51	0·40
4	28	9·26	13·55	4·29	12·88	0·33
	Av.	7·45	10·82	3·37	9·32	0·36
5	38	10·68	19·69	9·01	21·23	0·42
6	38	9·96	20·22	10·26	27·02	0·38
7	38	8·65	17·84	9·19	21·89	0·42
	Av.	9·76	19·25	9·49	23·38	0·41

The O_2/pyruvate ratio for dialysed pigeon brain suspensions in the presence and absence of added adenine nucleotide. Adenine nucleotide addition approximately doubles the pyruvate utilization and trebles the net O_2 uptake (cf. Tables 8 and 9). Thus it might be inferred that pyruvate oxidation is more complete in the presence of adenine nucleotide than in its absence. Table 11 fully confirms this expectation, since the O_2/pyruvate ratio averages 0·70 in the absence of added adenine nucleotide, and 0·96 in its presence. For that fraction of the pyruvate utilization which is specifically catalysed by adenine nucleotide, the molecular ratio, if it be considered separately, is 1·20 μmol. O_2/μmol. pyruvic acid. It should be noted that this value is much smaller than the corresponding figure obtained by McGowan [1937] for pyruvate oxidation in the minced tissue (O_2/pyruvate = 1·77).

Table 11. *The molecular ratio O_2/pyruvate for dialysed pigeon brain suspensions in the presence and absence of added adenine nucleotide* (A.N.)

Exp.	Net O_2 uptake μmol. Control	A.N.	Diff.	Pyruvic acid utilization μmol. Control	A.N.	Diff.	O_2/pyruvate Control	A.N.	Diff.
1	3·48	9·94	6·46	4·74	10·05	5·31	9·73	0·99	1·22
2	4·02	9·82	5·80	5·81	10·27	4·46	0·69	0·96	1·30
3	2·50	9·45	6·95	4·53	9·90	5·37	0·55	0·96	1·29
4	2·43	8·17	5·74	3·07	7·83	4·76	0·79	1·04	1·21
5	3·10	8·20	5·10	5·32	9·89	4·57	0·58	0·83	1·12
6	3·13	9·73	6·60	4·20	10·18	5·98	0·75	0·96	1·10
7	3·65	9·42	5·77	4·37	9·24	4·97	0·84	1·02	1·18
Av.	3·19	9·25	6·06	4·56	9·62	5·06	0·70	0·96	1·20

In considering the effect of adenine nucleotide on α-ketobutyrate oxidation (Table 9), it was concluded that after 3 hr. dialysis some adenine nucleotide still remains associated with the enzyme. Thus the O_2/pyruvate ratio would be much lower than 0·70 in the complete absence of adenine nucleotide, were it possible to realize this condition. Quite apart from this consideration, however, the mechanism of pyruvate oxidation is undoubtedly different in the presence, and in the absence, of adenine nucleotide, as will be shown later.

The O_2/α-ketobutyrate ratio for dialysed suspensions of pigeon brain. For washed preparations of minced pigeon brain, the O_2/α-ketobutyrate ratio (Table 10) averages about 0·39, a figure in agreement with previous work on the R.Q. for α-ketobutyrate oxidation under similar conditions. In Table 12 are recorded some corresponding values for this ratio, using dialysed pigeon brain suspensions. Series A (Table 12) refers to determinations carried out under the usual conditions. It will be seen that the O_2/α-ketobutyrate ratio is very similar to that obtained with the minced brain preparations.

It will also be noted, however, that if the brain is ground under rigorously ice-cold conditions, as recommended by Banga *et al.* [1939*b*], then the O_2/α-ketobutyrate ratio is consistently above 0·5 (series B, Table 12). This is the first definite indication that under certain conditions α-ketobutyrate can be oxidized to an extent greater than corresponds with oxidative decarboxylation. There seems to be little doubt that residual adenine nucleotide is responsible for this, since pyruvate oxidation

under the same conditions is very great in the absence of added adenine nucleotide, and addition of the latter does not produce the usual large catalytic effect (Table 13). A possible explanation is that adenine nucleotide exists in a combined form in the fresh brain, and that this combination, although stable under ice-cold conditions, breaks down at room temperature in the absence of an organized cell structure.

Table 12. *The O_2/α-ketobutyrate ratio for pigeon brain suspensions*

		O_2 uptake (μmol.)			α-Keto-butyric acid utilization μmol.	O_2/α-keto-butyrate
	Exp.	Con-trol	α-Keto-butyrate	Net		
Series A	1	2·36	4·08	1·72	4·16	0·41
	2	3·80	5·63	1·83	4·20	0·44
	3	2·21	3·13	0·92	2·57	0·36
	Av.	2·79	4·28	1·49	3·64	0·41
Series B	4	2·77	4·81	2·04	2·35	0·87
	5	3·30	5·73	2·43	4·26	0·57
	6	2·33	4·91	2·58	4·11	0·63
	7	2·67	5·57	2·90	5·09	0·57
	Av.	2·77	5·26	2·49	3·95	0·64

Table 13. *The O_2 uptake of dialysed pigeon brain suspensions, prepared under rigorously ice-cold conditions, respiring in pyruvic acid*

	O_2 uptake (μl./40 min.)					
	No adenine nucleotide added			Adenine nucleotide added		
Exp.	Con-trol	Pyruvate	Net	Con-trol	Pyruvate	Net
1	83·7	250·8	167·1	89·1	281·3	192·9
2	111·3	215·5	104·2	151·8	304·7	152·9
3	138·5	315·5	177·0	—	—	—
4	93·4	237·0	143·6	—	—	—
5	92·7	262·0	169·3	—	—	—
6	86·6	245·0	158·4	—	—	—
7	79·9	207·0	127·1	—	—	—
8	57·1	217·0	159·9	—	—	—
9	80·9	268·5	187·6	—	—	—
Av.	90·5	246·5	156·0	—	—	—
	(cf. 67·1	134·2	67·1 for 23 exps. under normal conditions (Tables 8 and 14))			

(5) *The R.Q. of dialysed pigeon brain suspensions in the presence of pyruvic and α-ketobutyric acids*

For washed preparations of minced pigeon brain, McGowan [1937] obtained an average value for the R.Q. in pyruvate of 1·30, after allowing for residual gas exchange. For the R.Q. of α-ketobutyrate oxidation under similar conditions, the average value 2·37 was obtained [Long & Peters, 1939]. Both figures agree well with other experimental facts.

As might be expected, the more complete oxidation of pyruvate by dialysed suspensions of pigeon brain which is observed in the presence of adenine nucleotide is accompanied by a lowered R.Q. Furthermore, the similarity between the O_2/α-ketobutyrate ratios for washed pigeon brain mince and dialysed suspensions under normal conditions, leads, as expected, to identical values for the R.Q. The R.Q. determinations were made by the indirect method of Long & Peters [1939].

The R.Q. for pyruvate oxidation by dialysed pigeon brain suspensions in the presence of inorganic phosphate and absence of added adenine nucleotide. Many factors contribute to the variation in the net O_2 uptakes observed with dialysed pigeon brain suspensions respiring in presence of pyruvate and inorganic phosphate only. The most important is the temperature of the grinding process, already mentioned: under ice-cold conditions high values were obtained. Other factors which tend to produce high values are (*a*) the use of less permeable dialysis sacs, (*b*) shorter duration of the dialysis, (*c*) shorter periods of grinding, and (*d*) lower temperature of dialysis. While (*b*) and (*c*) can easily be reproduced, it is more difficult to control (*a*) and (*d*); hence some scatter of the results is inevitable. The variable throughout appears to be the amount of residual adenine nucleotide still associated with the enzyme preparation.

Table 14. *The R.Q. for pyruvate oxidation by dialysed pigeon brain suspensions in the presence of inorganic phosphate only*

M/20 phosphate present throughout. 40 min. respiration in air at 28°.

	O_2 uptake (μl.)			CO_2 production (μl.)			R.Q.	
Exp.	Con-trol	Pyru-vate	Net	Con-trol	Pyru-vate	Net	Con-trol	Net
				Series A				
1	66·6	106·3	39·7	51·6	143·8	92·2	0·78	2·35
2	65·6	112·6	47·0	49·0	163·6	114·6	0·75	2·44
3	58·4	107·2	48·8	53·1	146·0	92·9	0·91	1·90
4	69·3	123·1	53·8	66·1	168·7	102·6	0·95	1·91
5	70·5	126·5	56·0	42·5	152·1	109·6	0·60	1·96
6	89·9	158·2	68·3	73·3	203·6	130·3	0·82	1·91
Av.	70·0	122·3	52·3	55·9	163·0	107·1	0·80	2·05
				Series B				
7	50·1	118·7	68·6	31·1	155·6	124·5	0·62	1·82
8	80·3	149·7	69·4	77·3	202·0	124·7	0·96	1·80
9	63·2	134·2	71·0	47·6	177·8	130·2	0·75	1·84
10	74·7	149·5	74·8	46·7	206·9	160·2	0·63	2·14
11	54·1	129·8	75·7	47·1	179·0	131·9	0·87	1·74
12	43·7	125·8	82·1	36·3	182·5	146·2	0·83	1·78
13	95·8	194·0	98·2	72·8	263·8	191·0	0·76	1·94
Av.	66·0	143·1	77·1	51·3	195·4	144·1	0·78	1·87

In Table 14 are recorded determinations of the R.Q. for pyruvate oxidation by dialysed pigeon brain suspensions, in the presence of phosphate and absence of added adenine nucleotide. Series A and B, respectively, include those experiments in which smaller and larger net O_2 uptakes have been observed. In Series A, a low value for the net O_2 uptake is accompanied by a high value for the net R.Q. In Series B, the higher average net O_2 uptake corresponds with a lower net R.Q. Since there is reason to believe that appreciable amounts of re-

sidual adenine nucleotide are active in Series B, the conclusion may be drawn that this activator is responsible for the lowering of the net R.Q. Complete confirmation for this is provided in the next section. The average residual gas exchange is the same in both Series A and B.

The R.Q. for pyruvate oxidation by dialysed pigeon brain suspensions in the presence of inorganic phosphate and adenine nucleotide. When excess adenine nucleotide is added to the enzyme system, the extent of scatter among a series of R.Q. determinations for pyruvate oxidation should be much reduced, since the most important variable, namely adenine nucleotide present in suboptimal concentration, is no longer operative. Table 15 shows the individual figures for eight determinations of the R.Q. under these conditions. The net O_2 uptake due to pyruvate oxidation is fairly constant, and amounts to nearly three times the average figure (64·7 μl.) observed in the absence of added adenine nucleotide (Table 14). The effect of adenine nucleotide is to lower the net R.Q. appreciably.

Table 15. *The R.Q. for pyruvate oxidation by dialysed pigeon brain suspensions in the presence of adenine nucleotide and inorganic phosphate*

$3 \times 10^{-4} M$ adenine nucleotide present throughout. Other conditions similar to those in Table 14.

	O_2 uptake (μl.)			CO_2 production (μl.)			R.Q.	
Exp.	Control	Pyruvate	Net	Control	Pyruvate	Net	Control	Net
1	63·5	254·0	190·5	45·7	366·7	321·0	0·72	1·69
2	68·4	264·6	196·2	47·1	383·6	336·5	0·69	1·72
3	103·9	296·5	192·6	73·9	380·7	306·8	0·70	1·59
4	90·2	270·3	180·1	66·7	361·5	294·8	0·74	1·64
5	122·9	284·0	161·1	87·7	364·7	277·0	0·71	1·72
6	81·0	257·5	176·5	52·2	345·5	293·3	0·65	1·66
7	125·0	316·5	191·5	79·9	399·2	319·3	0·64	1·66
8	112·0	288·5	176·5	79·2	361·3	282·1	0·71	1·60
Av.	95·9	279·0	183·1	66·6	370·4	303·8	0·69	1·66

The R.Q. for that part of the pyruvate oxidation in dialysed pigeon brain suspensions which is specifically catalysed by adenine nucleotide. In order to obtain the R.Q. for the adenine nucleotide-catalysed oxidation of pyruvate, it is best to determine the individual R.Q.'s in the presence, and in the absence, of added adenine nucleotide and of pyruvate, simultaneously on the same sample of dialysed enzyme. In practice this is difficult, since with duplicate estimations 24 Barcroft manometers would be required. The general method adopted here has been to use those results shown in Tables 14 and 15, which give the R.Q. values for pyruvate oxidation in the presence, and in the absence, of added adenine nucleotide. Although the R.Q. in the absence of added adenine nucleotide varies widely, the scatter among the individual values, presumably due to variable suboptimal quantities of adenine nucleotide in the enzyme preparation, should give the same net effect of adenine nucleotide for both series, A and B. This was the case (Table 16).

Table 16. *The R.Q. for that part of the pyruvate oxidation which is specifically catalysed by adenine nucleotide*

	Net O_2 uptake (μl.) due to pyruvate			Net CO_2 production (μl.) due to pyruvate			
	No addition	Added A.N.	Net	No addition	Added A.N.	Net	Net R.Q.
Series A	52·3	183·1	130·8	107·1	303·8	196·7	1·50
Series B	77·1		106·0	144·1		159·7	1·51
Av.	64·7	183·1	118·4	125·6	303·8	178·2	1·50

The R.Q. for α-ketobutyrate oxidation by dialysed pigeon brain suspensions. Under normal conditions, the net R.Q. for α-ketobutyrate oxidation by dialysed pigeon brain suspensions is identical with that previously found by Long & Peters [1939] for washed preparations of minced pigeon brain. The average value (2·38) for three determinations (Table 17, Series A) agrees closely with the figure 2·37 for minced brain. This is not unexpected, in view of the similarity of the ratio O_2/α-ketobutyrate for the two enzyme preparations.

When the R.Q. for α-ketobutyrate oxidation was determined with dialysed suspensions prepared under rigorously ice-cold conditions, it was found to be considerably lower than the normal value (Table 17, Series B). This is in accordance with the earlier observation that the O_2/α-ketobutyrate ratio is markedly increased under these conditions, as shown in Table 12.

Table 17. *The R.Q. for α-ketobutyrate oxidation by dialysed pigeon brain suspensions*

M/20 phosphate present throughout. 40 min. respiration at 28° in air.

	O_2 uptake (μl.)			CO_2 production (μl.)			R.Q.	
Exp.	Control	α-Ketobutyrate	Net	Control	α-Ketobutyrate	Net	Control	Net
				Series A				
1	85·0	125·7	40·7	59·1	156·0	96·9	0·70	2·38
2	69·7	104·1	34·4	60·5	135·2	74·7	0·87	2·17
3	76·5	115·3	38·8	52·0	151·4	99·4	0·68	2·56
Av.	77·0	115·0	38·0	57·2	147·5	90·3	0·74	2·38
				Series B				
4	59·7	124·7	65·0	34·9	143·6	108·2	0·59	1·67
5	48·2	96·5	48·3	21·1	106·2	85·1	0·44	1·76
Av.	53·9	110·6	56·7	28·0	124·7	96·7	0·52	1·71

(6) *The amounts of free and combined CO_2 formed during the oxidation of pyruvic acid by dialysed pigeon brain suspensions*

In the experiments already described on the determination of the R.Q. values for pyruvate oxida-

tion, the total CO_2 produced was obtained by the summation of the amounts of free and bound CO_2. The free CO_2 formed was estimated by the difference in gas absorption in the presence and absence of KOH-papers; the bound CO_2 was found by liberation of CO_2 by acid at the beginning and end of the respiration period. The usual controls in the absence of substrate were made throughout. It has been found that under any given set of experimental conditions both the total and relative amounts of free and bound CO_2 formed are remarkably constant, and that the omission of substrate, or the addition of adenine nucleotide, is accompanied by a new set of values (Table 18).

A pyruvate ion utilized by oxidative decarboxylation

$$CH_3CO.COO^- + \tfrac{1}{2}O_2 \rightarrow CH_3COO^- + CO_2$$

must be replaced by an acetate ion and 1 mol. free CO_2. A pyruvate ion completely oxidized,

$$CH_3CO.COO^- + 2\tfrac{1}{2}O_2 \rightarrow HCO_3^- + 2CO_2 + H_2O,$$

however, is replaced by a bicarbonate ion and 2 mol. CO_2. Thus in the first example all the CO_2 formed appears as gas, and in the second case two-thirds appear as gas and one-third as bicarbonate.

The experiments would best be performed in bicarbonate buffer saturated with O_2 containing 5% CO_2, but in fact the medium used was initially free from bicarbonate and the respiration was conducted in air. It has been observed, however, that during the equilibration period a considerable amount of bicarbonate is formed (about 70 and 40 μl. in the presence and absence of pyruvate, respectively). During the respiration period bicarbonate is formed much less rapidly, whereas the gaseous CO_2 formed is very considerable. Apparently equilibrium between the medium and its atmosphere has been reached at the beginning of the respiration period. Measurement of the amounts of CO_2 formed as gas and bicarbonate under different conditions are therefore possible; for pyruvate oxidation in the presence and absence of added adenine nucleotide the results are given in Table 18. The figures for that part of the pyruvate oxidation which is specifically catalysed by adenine nucleotide are shown in Table 19, from which it will be seen that about two-thirds of the CO_2 produced appears as gas (CO_2 gas formation) and one-third remains in solution (bicarbonate formation). These results are discussed later.

Table 18. *CO_2 distribution as gas and bicarbonate during the oxidation of pyruvate by dialysed suspensions of pigeon brain*

	Distribution of total CO_2 production (μl./40 min.)					
	CO_2 gas formation			Bicarbonate formation		
Exp.	Control	Pyruvate	Net	Control	Pyruvate	Net
	No adenine nucleotide added, Series A					
1	47·0	108·1	61·1	4·6	35·7	31·1
2	42·6	123·7	81·1	6·4	39·9	33·5
3	40·8	110·1	69·3	12·3	35·9	23·6
4	49·0	125·3	76·5	17·1	43·2	26·1
5	35·6	113·3	75·7	6·9	40·8	33·9
6	59·7	156·0	96·3	13·6	47·6	34·0
Av.	45·8	122·5	76·7	10·1	40·5	30·4
	No adenine nucleotide added, Series B					
7	26·7	115·7	89·0	4·4	39·9	35·5
8	58·3	147·2	88·9	19·0	54·8	35·8
9	41·2	132·7	91·5	6·4	45·1	38·7
10	45·6	156·3	110·7	1·1	50·6	49·5
11	42·6	138·3	95·7	4·5	40·7	36·2
12	33·8	144·5	110·7	2·5	38·0	35·5
13	56·1	186·1	130·0	16·7	77·7	61·0
Av.	43·5	145·9	102·4	7·8	49·5	41·7
	Adenine nucleotide added					
1	42·1	262·1	220·0	3·6	104·6	101·0
2	40·0	269·1	229·1	7·1	114·5	107·4
3	63·1	275·6	212·5	10·8	105·1	94·3
4	57·1	259·9	202·8	9·6	101·6	92·0
5	69·6	269·0	199·4	18·1	95·7	77·6
6	45·8	251·3	205·5	6·4	94·2	87·8
7	74·8	295·1	220·3	5·1	104·1	99·0
8	69·4	271·3	201·9	9·8	90·0	80·2
Av.	57·8	269·2	211·4	8·8	101·2	92·4

Table 19. *CO_2 production as gas and bicarbonate from that part of the pyruvate which is utilized specifically by adenine nucleotide (A.N.) catalysis*

	Distribution of CO_2 production (μl./40 min.)					
	CO_2 gas formation			Bicarbonate formation		
	No addition	Added A.N.	Net	No addition	Added A.N.	Net
Series A	76·7	211·4	134·7	30·4	92·4	62·0
Series B	102·4		109·0	41·7		50·7
Av.	89·5	211·4	121·9	36·1	92·4	56·3

DISCUSSION

The collective data presented in this paper, although still incomplete, allow a fairly detailed comparison to be made of the pyruvic acid oxidation processes occurring in minced and in ground pigeon brain. Knowledge of the behaviour of α-ketobutyric acid towards the same enzyme preparations has provided the initial clues to the problem. It has been shown (Table 6) that α-ketobutyric acid is equally rapidly utilized by minced and by ground pigeon brain under similar conditions. Furthermore, identical values have been obtained for the O_2/α-ketobutyrate ratio and the R.Q. for the two enzyme preparations. It therefore seems justifiable to draw the conclusion that those enzymes responsible for oxidative decarboxylation and anaerobic dismutation, reactions which are known to utilize α-ketobutyric acid in minced brain, are also operative in the ground tissue.

Pyruvic and α-ketobutyric acids have previously been shown [Long, 1942] to be equally rapidly utilized by respiring minced pigeon brain. In

ground preparations (suspensions), on the other hand, pyruvate has now been found to be more rapidly utilized than α-ketobutyrate. This observation can be explained in the following manner. It has been shown in the experimental section that under certain conditions, such as the rigorously ice-cold grinding of the brain, the tissue preparation contains appreciable amounts of adenine nucleotide, even after 3 hr. dialysis, and it is probable that even under the most favourable conditions obtainable, suboptimal amounts of adenine nucleotide are always associated with the tissue enzyme. This fact becomes important when it is borne in mind that pyruvate utilization by ground preparations of pigeon brain is very susceptible to catalytic amounts of adenine nucleotide (Table 9), whereas α-ketobutyrate utilization is not nearly so sensitive, a definite effect in this case being difficult to detect. It seems certain, therefore, that if adenine nucleotide could be excluded completely from the system, pyruvic acid utilization would be much lower than that normally observed, and pyruvic and α-ketobutyric acids would probably be equally rapidly utilized. When the ground tissue enzyme was thoroughly extracted with hypotonic saline, as in the anaerobic oxidation experiments, the reduction time of methylene blue was found to be the same with either pyruvate or α-ketobutyrate as substrate (Tables 1 and 2). This is evidence in favour of equal rates of oxidation of pyruvic and α-ketobutyric acids by ground preparations of pigeon brain in the presence of a phosphate buffer and in the complete absence of adenine nucleotide.

The reactions involved in the oxidation of pyruvic acid by dialysed pigeon brain suspensions, respiring in phosphate buffer, must now be considered. McGowan [1937], using minced pigeon brain, found the O_2/pyruvate ratio to be 1·77 mol. O_2 absorbed/mol. pyruvic acid utilized. The corresponding ratio for dialysed suspensions, under comparable conditions, has been found to be 0·70 (Table 11). This indicates that pyruvate is much less completely oxidized by ground brain suspensions. Even this value, however, is too high, since some of the pyruvate is utilized by residual adenine nucleotide catalysis, which requires 1·20 mol. O_2/mol. pyruvic acid. The true figure in the complete absence of adenine nucleotide is therefore probably much lower, and is no doubt very similar to the O_2/α-ketobutyrate ratio (0·41). This is confirmed by the net R.Q. values. McGowan [1937] reported the R.Q. for pyruvate oxidation in minced brain to be 1·30. In this work the corresponding figure for dialysed suspensions was found to vary between 1·74 and 2·44. Furthermore, low values for the net O_2 uptake due to pyruvate oxidation are accompanied by high R.Q. values. Allowing for the pyruvate which is oxidized by residual adenine nucleotide catalysis, the true R.Q. in the complete absence of adenine nucleotide would probably be similar to the R.Q. for α-ketobutyrate oxidation under the same conditions (2·38). Both lines of evidence strongly suggest that in the complete absence of adenine nucleotide, pyruvate and α-ketobutyrate oxidations would proceed according to the same reactions, i.e. oxidative decarboxylation and anaerobic dismutation. Presumably the process of grinding the brain tissue, and the consequent destruction of the cell structure, alters the conditions necessary for the complete oxidation of pyruvate to CO_2 and water. The latter occurs to the extent of about 70 % of the pyruvate utilized by respiring minced brain [Long, 1938]. This fraction of the pyruvate which disappears is probably utilized by the alternative process of oxidative decarboxylation when the oxidation is carried out in the presence of ground preparations of pigeon brain.

If these deductions are correct, namely that dialysed pigeon brain suspensions, respiring in a phosphate-buffered inorganic medium, utilize pyruvic acid by oxidative decarboxylation and anaerobic dismutation exclusively, pyruvic acid certainly undergoes a totally different type of oxidation in the presence of adenine nucleotide. So far there is no evidence that adenine nucleotide exerts any influence on either of these reactions, and presumably adenine nucleotide opens up a new reaction path for pyruvic acid. This view is supported by the increased utilization of pyruvic acid when adenine nucleotide is added, and the completely different set of values obtained for the O_2/pyruvate ratio and the R.Q. for that part of the pyruvate oxidation which is specifically catalysed by adenine nucleotide.

If that part of the pyruvate utilization which is specifically catalysed by adenine nucleotide only be considered, the O_2/pyruvate ratio of 1·20 and R.Q. value of 1·50 indicate that 1·80 mol. CO_2 are produced by the oxidation of 1 mol. pyruvic acid, i.e. 60 % of the carbon of pyruvic acid is converted into CO_2. So far then the reaction can be expressed in the form

$$5CH_3.CO.COOH + 6O_2 \rightarrow 9CO_2 + (6C + 20H + 9O).$$

Further, if the CO_2 produced in the form of bicarbonate is one-third of the total CO_2 (Table 19), then 6 mol. CO_2 are formed as gas and 3 mol. as bicarbonate. In order to balance the total acidic groups taking part in the reaction, 2 mol. fixed acid must also be produced:

$$5CH_3.CO.COOH + 6O_2 \rightarrow \underset{(CO_2 \text{ gas formation})}{6CO_2} + \underset{(\text{bicarbonate formation})}{3CO_2} + \underset{(2 \text{ equiv. fixed acid})}{(6C + 20H + 9O)}.$$

The constitution of the fixed acid or acids is not known. The three most likely possibilities are that

there are (1) two 3-carbon monobasic acids, (2) one 2-carbon and one 4-carbon monobasic acid, (3) one 6-carbon dibasic acid. Allowing for the 4 or 5 mol. water formed, there are various possibilities, which would fit the equation perfectly. Speculation is here of little value, and characterization and determination of the fixed acids must be awaited.

SUMMARY

1. Inorganic phosphate or arsenate is required for the anaerobic oxidation of both pyruvate and α-ketobutyrate by ground preparations of pigeon brain with methylene blue as H-acceptor.

2. Inorganic phosphate stimulates the O_2 uptake and the utilization of pyruvate and of α-ketobutyrate by respiring preparations of ground pigeon brain (suspensions). Arsenate is slightly inhibitory.

3. α-Ketobutyrate is utilized at the same rate under comparable conditions by minced and ground preparations of pigeon brain. Pyruvate is more rapidly utilized by ground than by minced brain. This apparent discrepancy is discussed.

4. Adenine nucleotide markedly increases the net O_2 uptake and the pyruvate utilization of pigeon brain suspensions when inorganic phosphate is present. It stimulates α-ketobutyrate oxidation under certain conditions, but normally its influence is difficult to detect.

5. Normally the O_2/α-ketobutyrate ratio and the R.Q. for α-ketobutyrate oxidation is the same for brain suspensions as for minced brain.

6. The O_2/pyruvate ratio, R.Q. and CO_2 distribution as gas and bicarbonate have been determined in the presence and absence of added adenine nucleotide. For that part of the pyruvate oxidation specifically catalysed by adenine nucleotide, the following values have been obtained: O_2/pyruvate ratio, 1·20; R.Q. 1·50; gaseous CO_2/bicarbonate CO_2, 2·0.

7. The mechanisms of pyruvate oxidation in minced and ground pigeon brain preparations are compared. The mode of oxidation of pyruvate by pigeon brain suspensions in the presence of inorganic phosphate and adenine nucleotide is discussed.

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The Biological Value of Mixed Proteins in Food Served in Royal Air Force Airmen's Messes

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While much information is available on the biological value of isolated proteins and of mixed proteins of individual foodstuffs, relatively little is known about the biological value of the proteins (nitrogen) of mixed human dietaries. Supplementary relationships between proteins make it naturally impossible to calculate such values from those of separate constituents. Hence an assessment of a whole diet is of greater practical importance than the evaluation of the contribution of its different components.

The experiments described below were carried out to determine the biological value of the combined proteins contained in the food served to airmen at four Royal Air Force Stations in this country during October–December, 1941.

EXPERIMENTAL

At each Station the total food available to 5 airmen at all meals during 1 week was collected. Care was taken to ensure that this was representative of that served in the messes. The airmen queued for their meals and samples were obtained by taking at random 5 plates offered to the men as they filed past. The food was collected in galvanized pails. Tea and cocoa, as served, were not added, since the large amount of water would have caused difficulties during processing. The tinned milk, sugar and cocoa powder used in the making of the tea and cocoa, however, were added.

The food was then stored at $-20°$ for 7 days. The total food was ground in the frozen state, the temperature not being allowed to exceed $-5°$. Care had to be taken to avoid the addition of large pieces of fat, and it was essential to spread the butter and margarine on the bread, otherwise these fats clogged the mill. The ground, frozen material which was snow-like in appearance was mixed and dried in an oven maintained at 70° and through which a rapid current of air was passed, for 6–7 hr. Except during the last ½ hr., the temperature of the food was much lower than that of the oven, owing to the evaporation of water. The dried food was then minced in a sausage mincer and finally ground in a coffee mill; the resulting product was a fine brown-coloured powder, stable and suitable for analyses. The total weight of the dried food was determined and from it the average weight of dried diet representing one man's daily ration was calculated. The analyses of these dried diets for the total amounts of nutrients have been carried out and will be reported later.

The average amount of food left unconsumed was also ascertained. Table 1 gives the amount of protein in the food served and that in the food consumed. Protein values were obtained by multiplication of the nitrogen values by the factor 6·25.

Table 1. *Amounts of protein offered to and consumed by airmen (average/man daily)*

	Station 1 (diet I)	Station 2 (diet II)	Station 3 (diet III)	Station 4 (diet IV)
Protein in diet (%)	14·6	16·0	16·8	17·2
Protein offered (g.)	86·4	105·2	106·3	100·8
'Plate waste' (%)	3·3	6·0	5·3	7·8
Protein consumed (g.)	83·6	98·9	100·7	92·9

The amounts of the raw foodstuffs used in the different cookhouses were known. From tables of food values it was, therefore, possible to calculate the percentages of protein contributed to the dietaries by the individual foodstuffs. It was also possible to make a rough estimate of the amounts of the proteins which were of vegetable and animal origin respectively (Table 2).

The biological values and true digestibilities of diets from the four R.A.F. stations were determined by the method of Mitchell [Mitchell, 1924; Mitchell & Carman, 1926], using rats as experimental animals. The diets were diluted with starch to give an 8% level of protein intake (N × 6·25), and sufficient margarine fat was added to bring the total fat content of each diet to 10%. As analysis indicated that the resulting diets were rather low in Ca, 1·5% $CaHPO_4.2H_2O$ was added to each. Table 3 gives the composition and analysis of the experimental diets.

Table 2. *Percentages of total protein contributed by individual foodstuffs in dietary. Calculated total percentage of protein of animal origin*

Foodstuffs	Station 1	Station 2	Station 3	Station 4
Bread, white	22·8	25·4	22·0	20·1
Bread, National	6·7	10·8	6·9	8·1
Flour, white	5·2	4·2	4·1	4·2
Oatmeal	2·7	1·1	1·5	2·3
Rice	0·9	0·9	0·5	0·3
Custard powder	0·1	0·1	—	0·1
Shredded wheat	0·4	—	0·2	—
Cornflakes	0·3	1·2	0·5	0·2
Cake	3·1	—	3·4	—
Spongecake	0·9	—	—	—
Fruit pie	—	—	0·2	—
Jam	0·1	—	—	0·1
Milk	5·0	3·9	6·8	3·4
Cheese	4·2	2·8	2·7	3·9
Butter	0·1	—	0·1	0·1
Beef	11·1	14·8	12·3	5·6
Boneless beef	4·6	—	—	6·2
Mutton	—	3·0	—	4·9
Preserved meat	3·9	—	3·8	3·6
Liver	—	—	3·8	2·5
Kidney	2·6	5·3	2·5	—
Sausage	2·4	3·9	6·1	5·5
Oxtail	—	—	0·4	—
Oxheart	—	—	—	1·9
Meat roll	1·2	4·2	0·8	1·2
Meat paste	1·2	—	—	—
Meat pie	—	—	—	4·7
Pork pie	—	—	1·3	—
Cornish pastie	—	—	3·4	—
Sausage roll	—	—	1·3	—
Bacon	4·7	4·3	3·6	3·1
Fish (haddock)	3·8	4·0	—	5·5
Kippers	—	—	—	2·5
Fish cake	—	—	1·1	—
Potatoes	6·3	5·9	6·4	7·0
Cabbage	1·5	0·8	0·4	0·4
Brussels sprouts	—	—	0·2	0·4
Cauliflower	—	0·4	—	0·4
Carrot	0·4	0·2	0·3	0·5
Turnip	0·3	0·5	0·3	0·3
Parsnip	—	—	0·3	0·3
Celery	—	—	0·1	—
Beetroot	0·2	0·2	0·1	—
Onion	—	0·1	—	0·1
Lettuce	0·1	—	—	—
Tomatoes	0·1	0·1	0·1	0·1
Dried beans	1·3	0·5	1·4	—
Lentils	—	—	0·1	—
Canned beans	1·1	0·8	0·3	—
Apples	—	0·2	0·1	0·1
Prunes	—	0·1	—	—
Sultanas	0·2	—	—	0·2
Raisins	—	—	0·2	—
Cocoa	2·0	1·0	1·1	1·0
Percentage of total protein of animal origin (calc.)	43·2	42·2	42·7	44·7

Three groups of four litter-mate female rats 25–30 days old and weighing 52–71 g. were placed in glass and stainless steel metabolic cages. A description of the cages and of the experimental technique is given elsewhere [Henry, Kon & Watson, 1937]. Each animal received each of the diets in turn. The diets were fed mixed with 1½ times their weight of water.

Table 3. *Composition and analysis of experimental diets*

R.A.F. diet no. ...	I	II	III	IV
R.A.F. diet (%)	54·7	49·9	47·8	46·5
Margarine fat (%)	1·6	1·1	1·6	3·2
Potato starch (%)	10·0	10·0	10·0	10·0
$CaHPO_4.2H_2O$ (%)	1·5	1·5	1·5	1·5
Rice starch (%)	32·2	37·5	39·1	38·8
Nitrogen (%)	1·329	1·313	1·308	1·297
Moisture (%)	10·96	10·62	10·46	10·83

RESULTS

The results of the rat tests are shown in Tables 4 and 5. All four diets have a high biological value and a high degree of digestibility. The differences between them are not large, the extremes for biological value being 77·6 for diet III and 81·2 for diet IV, and for true digestibility 85·1 and 88·5 for diets I and IV respectively. Statistical analysis (Table 6) of the results by the paired *t*-test method ['Student', 1908; 1925] shows that the biological values of the proteins of diets II and IV are significantly superior to those of diet III but that other differences can be accounted for by errors of sampling. With one exception the order of ascending biological values is the same as that of ascending true digestibilities. When the two values are multiplied one by the other, one of the steps in calculation of the 'protein value' of a food [Mitchell, 1927; Mitchell & Hamilton, 1929], and the resultant figures are submitted to statistical analysis, diet IV again proves better than diet III but the other differences are without significance. When the 'protein values' of the four diets are calculated on the dry basis the following values are obtained: diet I, 9·5; diet II, 11·1; diet III, 11·1; and diet IV, 12·2. Here again diet IV proves best as it is highest in biological value, true digestibility and nitrogen

Table 4. *Individual nitrogen metabolism data*

Rat no.	R.A.F. diet tested	Wt. of rat, g. Initial	Wt. of rat, g. Final	Diet intake g.	N intake mg.	Faecal N mg.	Urinary N mg.	Biological value	True digestibility	Biological value × true digestibility
				Low egg-nitrogen diet						
1	—	62	73	49·33	—	90·6	98·0	—	—	—
2	—	70	72	43·08	—	109·5	87·1	—	—	—
3	—	66	71	41·65	—	92·3	95·0	—	—	—
4	—	69	76	50·62	—	109·6	88·4	—	—	—
5	—	63	66	39·37	—	96·2	81·6	—	—	—
6	—	62	68	49·64	—	107·6	87·3	—	—	—
7	—	58	65	45·84	—	91·1	75·4	—	—	—
8	—	59	66	47·50	—	103·0	80·8	—	—	—
9	—	60	67	48·74	—	100·3	61·2	—	—	—
10	—	58	62	43·35	—	98·3	68·3	—	—	—
11	—	55	61	44·13	—	94·1	68·6	—	—	—
12	—	55	63	48·38	—	100·0	68·6	—	—	—
				First experimental period						
1	I	74	89	56·98	757·3	215·5	266·4	76·9	87·3	67·1
2	II	75	90	57·00	748·5	241·9	234·2	79·2	87·0	69·0
3	III	71	84	49·00	640·4	185·7	228·1	77·1	88·4	68·2
4	IV	81	94	57·45	745·1	204·1	222·3	82·1	90·0	73·9
5	I	70	77	39·98	531·5	170·7	196·0	76·8	86·6	66·4
6	II	70	83	53·93	708·1	219·8	212·6	81·2	86·5	70·2
7	III	70	82	54·49	712·5	220·1	225·8	78·1	84·7	65·4
8	IV	71	85	56·42	732·0	211·0	225·3	79·9	88·3	70·6
9	I	72	88	56·97	757·1	210·5	233·1	76·4	89·3	68·2
10	II	67	84	57·00	748·5	204·0	197·5	82·5	90·2	74·4
11	III	69	85	57·47	751·5	195·5	227·6	79·1	91·6	72·5
12	IV	72	89	57·46	745·3	202·7	203·3	82·6	89·8	74·2
				Second experimental period						
1	IV	102	123	76·45	991·6	273·0	298·8	83·4	90·7	75·6
2	I	104	124	73·49	976·8	291·2	281·2	83·0	89·3	74·1
3	II	97	114	65·94	866·0	257·7	282·5	78·9	88·1	69·5
4	III	107	119	67·42	881·6	293·6	323·2	73·6	84·6	62·3
5	IV	85	102	59·87	776·5	232·2	221·6	83·7	89·5	74·9
6	I	95	110	71·96	777·5	285·6	252·5	80·4	69·7	56·0
7	II	95	110	66·47	873·0	240·4	246·0	84·6	88·4	74·8
8	III	95	108	65·95	862·3	271·2	301·1	75·5	85·9	64·8
9	IV	102	122	75·99	985·6	273·0	237·9	84·5	91·6	77·3
10	I	98	116	75·39	1002·0	318·1	245·6	83·1	85·6	71·2
11	II	99	116	72·97	958·0	323·0	248·8	82·7	85·1	70·4
12	III	101	118	76·00	993·9	288·6	285·3	79·9	88·9	71·1

Table 4 (*cont.*)

Rat no.	R.A.F. diet tested	Wt. of rat, g. Initial	Wt. of rat, g. Final	Diet intake g.	N intake mg.	Faecal N mg.	Urinary N mg.	Biological value	True digestibility	Biological value × true digestibility
				Third experimental period						
1	III	134	152	87·48	1144·0	347·8	405·3	78·1	89·6	69·9
2	IV	135	155	85·49	1109·0	332·9	344·2	81·7	89·6	73·2
3	I	125	143	78·47	1043·0	341·6	284·6	83·6	85·3	71·3
4	II	130	148	76·46	1004·0	285·9	293·2	83·9	90·1	75·6
5	III	112	132	68·60	897·0	282·5	259·5	84·2	88·1	74·2
6	IV	122	146	83·49	1083·0	297·0	308·4	83·9	92·3	77·5
7	I	120	128	60·16	799·7	248·4	280·2	81·6	85·0	69·4
8	II	129	136	73·48	965·0	309·2	235·8	77·2	85·6	66·1
9	III	131	153	86·99	1137·0	356·1	308·9	81·2	89·5	72·6
10	IV	127	150	86·99	1128·0	350·8	297·4	81·6	86·8	70·8
11	I	125	150	85·00	1130·0	336·6	283·5	84·5	90·1	76·1
12	II	127	151	87·50	1149·0	357·2	292·9	83·2	87·9	73·1
				Fourth experimental period						
1	II	163	173	86·40	1135·0	439·5	403·1	78·6	83·3	65·4
2	III	164	179	93·87	1225·0	437·7	457·5	73·6	83·6	61·5
3	IV	150	164	84·51	1096·0	378·1	396·8	72·8	84·4	61·4
4	I	156	169	83·32	1107·0	375·9	390·2	76·1	85·1	64·8
5	II	137	152	76·49	1005·0	319·4	343·1	78·4	88·1	69·1
6	III	153	161	82·74	1082·0	367·9	409·7	73·4	86·6	63·5
7	IV	132	138	60·43	783·8	253·7	318·8	77·5	84·5	65·5
8	I	141	147	72·38	762·0	355·7	379·6	70·8	80·8	57·2
9	II	168	187	94·82	1245·0	435·1	352·3	80·4	87·5	70·3
10	III	162	183	97·45	1274·0	479·0	363·2	77·1	80·4	61·9
11	IV	163	180	96·00	1245·0	456·7	346·0	80·6	84·9	68·4
12	I	162	178	97·47	1297·0	419·9	372·3	79·1	87·5	69·2
				Low egg-nitrogen diet						
1	—	170	164	46·86	—	148·0	189·3	—	—	—
2	—	173	172	56·77	—	144·3	181·6	—	—	—
3	—	161	167	68·42	—	171·2	132·5	—	—	—
4	—	169	170	60·92	—	160·2	164·2	—	—	—
5	—	146	140	36·29	—	96·4	143·3	—	—	—
6	—	157	153	49·44	—	139·5	145·2	—	—	—
7	—	133	142	59·85	—	135·0	175·3	—	—	—
8	—	143	149	60·76	—	147·9	145·7	—	—	—
9	—	178	170	52·35	—	164·9	128·8	—	—	—
10	—	182	179	58·92	—	140·6	147·1	—	—	—
11	—	183	181	64·44	—	193·1	131·9	—	—	—
12	—	181	182	70·87	—	195·9	129·1	—	—	—

Table 5. *Mean data for biological values and true digestibilities of the diets studied*

R.A.F. diet no.	Period I	Period II	Period III	Period IV	Mean	Standard error of mean
			Biological value			
I	76·7	82·2	83·2	75·3	79·4	±1·18
II	81·0	82·1	81·4	79·1	80·9	±0·71
III	78·1	76·3	81·2	74·7	77·6	±0·95
IV	81·5	83·9	82·4	77·0	81·2	±0·95
			True digestibility			
I	87·7	81·5	86·8	84·5	85·1	±1·58
II	87·9	87·2	87·9	86·3	87·3	±0·57
III	88·2	86·5	89·1	83·5	86·8	±0·91
IV	89·4	90·6	89·6	84·6	88·5	±0·80
			Biological value × true digestibility			
I	67·2	67·1	72·3	63·7	67·6	±1·74
II	71·2	71·6	71·6	68·3	70·7	±0·94
III	68·7	66·1	72·2	62·3	67·3	±1·34
IV	72·9	75·9	73·8	65·1	71·9	±1·41

Table 6. *Statistical analysis of the data in Table 5*

Difference tested	Diet I–II	Diet I–III	Diet I–IV	Diet II–III	Diet II–IV	Diet III–IV
			Biological value			
Numerical difference	−1·5	+1·8	−1·8	+3·3	−0·3	−3·6
Standard error of mean	±1·11	±1·58	±1·77	±1·23	±1·18	±1·23
*P**	1 : 5	1 : 4	1 : 3	1 : 47	1 : 1	1 : 72
	Not sig.	Not sig.	Not sig.	Significant	Not sig.	Significant
			True digestibility			
Numerical difference	−2·2	−1·7	−3·4	+0·5	−1·2	−1·7
Standard error of mean	±1·66	±1·65	±1·97	±1·34	±1·06	±1·16
*P**	1 : 5	1 : 3	1 : 9	1 : 1	1 : 3	1 : 6
	Not sig.	Not sig.	Not sig.	Not sig.	Not sig.	Not sig.
			Biological value × true digestibility			
Numerical difference	−3·1	+0·3	−4·3	+3·4	−1·2	−4·6
Standard error of mean	±1·75	±1·94	±2·64	±1·85	±1·81	±1·85
*P**	1 : 10	1 : 1	1 : 8	1 : 11	1 : 2	1 : 33
	Not sig.	Not sig.	Not sig.	Not sig.	Not sig.	Significant

* *P* = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

content. Diet I takes last place, while the superiority in biological value of diet II over diet III is compensated for by the higher nitrogen content of III.

DISCUSSION

It is of interest to compare the data for the complete R.A.F. diets with the values reported in the literature for separate proteins, or for their combinations. Our results were obtained with young growing animals and therefore the combined requirements for growth and maintenance were measured. The work of Mitchell [1924] has shown that with growing rats the size of the animal has little effect on the estimate of the biological value of a protein. There are, however, indications [Sumner, 1938; French & Mattill, 1935; also unpublished work from one of our laboratories] that with adult rats the results are different from those with rats in the vigorous period of growth. Also, as the biological value of a protein varies according to the level of intake, only corresponding levels should be compared. The biological values of the R.A.F. diet proteins are high. Of single foodstuffs they are surpassed only by eggs and milk and nearly equalled by liver and kidney.* Of the proteins of vegetable products none can be said to attain these values. There must have been much supplementation of the poorer proteins by the better.

Relatively few estimations of combinations of foodstuffs have been published, and hardly any of them deal with complete mixed diets. Most come from India and China. Adolph & Cheng [1935] reported a very high biological value, 83 at a 10% level, for a mixture of maize, millet and soya bean, but Lan [1936] obtained a lower value, 73, for the same mixture. He quotes a value of 77, however, for a combination of soya bean and wheat gluten. Swaminathan [1938], using adult rats, investigated various mixtures of vegetable origin and obtained the highest values, 84 and 81 respectively, for combinations of red and green gram, *Cajanus indicus*, *Phaseolus radiatus* with ragi, *Eleusine coracana*. A mixed vegetarian diet of rice, red gram, black gram, amaranth leaves, brinjal and coconut yielded in his hands [Swaminathan, 1937] at a 10% level of intake, figures for biological value and true digestibility of 82 and 92 respectively. The supplementary relationship between animal and vegetable proteins is well known [Mitchell, 1924; Mitchell & Carman, 1926], and the value of an addition of milk has been confirmed by Swaminathan [1938]. He thus attained values as high as 84–94 for combinations of approximately 10 parts of ragi with 2 parts of various pulses and 1 part of dry skim milk, and values from 77 to 90 for similar combinations of rice, pulse and milk. There is no doubt that biological values as high and even higher than those now reported can be obtained for predominantly vegetarian diets. However, the 'protein value' of such diets works out at a much lower figure than those for the R.A.F. diets. Thus Swaminathan's [1937] diet, which is very similar to the 'Poor Madrassi' diet of Aykroyd & Krishnan [1937], contained only 10·5% crude protein as against nearly double this figure for the R.A.F. diets. Its 'protein value' is therefore 6·6—about half that of the best R.A.F. diet.

SUMMARY

1. A method is described whereby the total food served at meals may be converted into a homogeneous and stable product, suitable for analyses to determine its nutritive value.

2. The biological values of the mixed proteins contained in the food served at four R.A.F. Stations

* du Toit & Smuts [1941] have very recently reported a biological value of 94 for white fishmeal.

were 79·4, 80·9, 77·6 and 81·2 respectively. The mixture of proteins in all diets had, therefore, a high biological value which must have been due to supplementation amongst the proteins. Not much more than 40 % of the total proteins in the diets were of animal origin.

3. The true digestibility coefficients of the four diets were 84·5, 86·3, 83·5 and 84·6 respectively.

We are indebted to Dr F. Kidd and his staff at the Low Temperature Research Station, Cambridge, for their technical advice in the processing of the food and also for hospitality. Through the courtesy of Dr H. Chick of the Lister Institute, much help was given by Mr G. A. Childs (now Sergeant in the Royal Air Force). We wish to acknowledge the help of Miss B. Cash in the carrying out of the nitrogen metabolism analyses.

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Studies on the Acetone-Butanol Fermentation

4. ACETOACETIC ACID DECARBOXYLASE OF *CL. ACETOBUTYLICUM* (BY)

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(*Received 7 December 1942*)

Although the enzyme systems responsible for the fermentation of pyruvic acid and of glucose by *Cl. acetobutylicum* are so unstable as a whole that, in the absence of the substrate, their activity cannot be preserved in cell suspensions for more than a few hours [Davies, 1942], one enzyme has been found to be extremely stable; this is acetoacetic acid decarboxylase, the enzyme responsible for the decarboxylation of acetoacetic acid to acetone. The quantitative nature of the reaction, its optimum *p*H, and the effect of the age of the cells on their decarboxylase activity, have already been described; also the insensitivity of the enzyme to O_2, its ability to withstand acetone, and the fact that it can be extracted cell-free from the acetone powder [Davies, 1942]. In this paper the purification and properties of the isolated enzyme will be described. The parallel isolation of the lysine decarboxylase of *Escherichia coli* will be published later [Gale & Epps, 1943].

METHODS

Preparation of acetoacetic acid. 13 g. of redistilled ethyl acetoacetate were dissolved in 100 ml. *N* NaOH and kept at room temperature for 40 hr. The ice-cooled solution was acidified with H_2SO_4 (25 ml. of 20 % by vol.); the acetoacetic acid was twice extracted with 150 ml. ether and the ether evaporated at room temperature. From the residue neutralized with *N* NaOH the unchanged ester was removed by extraction with ether; finally the ether was evaporated *in vacuo*. After standardization by measuring the CO_2 evolved when a portion was incubated with acetone-treated cells of *Cl. acetobutylicum* in Warburg manometers, the concentration was adjusted to *M*/2.

Preparation of acetone-treated cells. 5 l. flasks containing 4½ l. of 2 % glucose-liver medium [Davies & Stephenson, 1941] were sown with 80 ml. of a vigorously fermenting culture of *Cl. acetobutylicum* (BY) on 5 % maize-meal mash, and were then incubated at 37° until the Rothera acetone test was intense (about 40 hr.). The culture was then strained through muslin and the cells collected in a Sharples super-centrifuge. They were washed once on the ordinary centrifuge with about 400 ml. distilled water and suspended in 50–100 ml. distilled water. This suspension was blown rapidly from a pipette into 10 vol. of vigorously stirred acetone; after a few minutes the precipitate was collected on a

Buchner funnel, washed with acetone and with ether, and sucked dry.

Conditions for the determination of enzyme activity

The optimum substrate concentration, and the optimum *p*H for decarboxylation, were determined for both fresh and acetone-treated cells. Q_{CO_2} for the decarboxylation was measured by Warburg manometers.

Effect of substrate concentration. It is seen (Fig. 1) that the affinity of the enzyme for its substrate is fairly low, and is slightly lower for fresh cells than

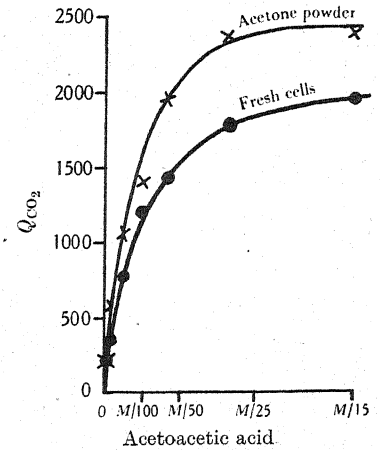

Fig. 1. Effect of substrate concentration on the rate of decarboxylation by fresh and acetone-treated cells. Warburg manometers contained 0·3 ml. 2*M* acetate buffer *p*H 4·5 (for fresh cells) or *p*H 5·0 (for acetone-treated cells), sufficient *M*/2 acetoacetic acid to give concentrations of *M*/15 to *M*/1000, and in the side bulb 0·1 ml. cell suspension; total vol. 3·0 ml.; temp. 37·5°; gas phase air.

for acetone-treated cells, which require *M*/20 acetoacetic acid to saturate the enzyme, the half-speed concentration being 0·007*M*.

Effect of p*H*. This is shown in Fig. 2; curves 1 and 2 were obtained with two different suspensions of fresh cells and the optimum *p*H differs considerably for the two. The optimum *p*H for certain amino-acid decarboxylases of other micro-organisms also varies similarly [Gale, personal communication]. The optimum *p*H for the decarboxylation by acetone-treated cells is seen to be 5·0.

Estimation of the activity of the enzyme preparations. Conditions of optimum activity were used, the *p*H being 5·0 and the final acetoacetic acid concentration $< M/20$. Decarboxylation was measured in Warburg manometers containing in a total volume of 3·0 ml., 0·3 ml. 2*M* acetate buffer of *p*H 5·0 and 0·5 ml. *M*/2 acetoacetic acid; the gas space contained air and the temperature was 37·5°.

The rate of CO_2 evolution has been correlated with the C content of the enzyme preparations, determined by the method of Van Slyke & Folch [1940], and the activities are expressed as μl. CO_2/hr. × mg. C, called $Q^C_{CO_2}$. One enzyme unit (E.U.) has

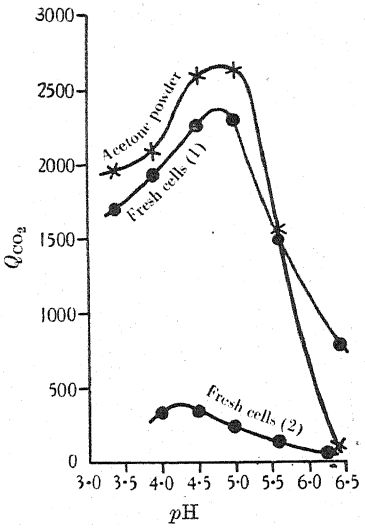

Fig. 2. Effect of *p*H on the rate of decarboxylation by fresh and acetone-treated cells. Warburg manometers contained 0·3 ml. 2*M* acetate buffer of the desired *p*H, 0·5 ml. *M*/2 Na acetoacetate, and in the side bulb 0·1 ml. cell suspension (for fresh cells 4·4 mg. dry wt. cells/ml.; for acetone-treated cells 5 mg. acetone powder/ml.); total vol. 3·0 ml.; temp. 37·5°; gas phase air.

been defined as the amount which will liberate 100 μl. CO_2 in 5 min. at 37·5° under the optimum conditions.

Extraction of the enzyme

Distilled water at 37° was the most satisfactory extractant. The *p*H was not critical but at *p*H 4·0 extraction became very poor (Table 1). Prolonged

Table 1. *Extraction of the enzyme*

5 mg. of acetone-treated cells were incubated for 4 hr. at 37° with 5 ml. of the following: (*a*) distilled water, (*b*) 0·9% NaCl, (*c*) *M*/100 acetate *p*H 4·0, (*d*) *M*/100 acetate *p*H 5·0, (*e*) *M*/100 acetate *p*H 6·0, (*f*) *M*/75 phosphate *p*H 7·0, (*g*) *M*/100 borate *p*H 8·0, (*h*) *M*/100 borate *p*H 9·0. The suspensions were then centrifuged and a suitable portion of the supernatant tested for decarboxylase activity.

Extracting agent	Water	0·9% NaCl	Buffer at *p*H 4·0	5·0	6·0	7·0	8·0	9·0
% initial activity extracted in 4 hr. at 37°	52	41	6	51	50	38	29	25

incubation results in bacterial contamination of the acetone powder suspension, hence a few drops of capryl alcohol were added to one half the suspension to check this; it will be seen that whereas in

24 hr. 95% of the enzyme was extracted in the presence of capryl alcohol, 140% was extracted in its absence (Fig. 3). This extra enzyme could hardly

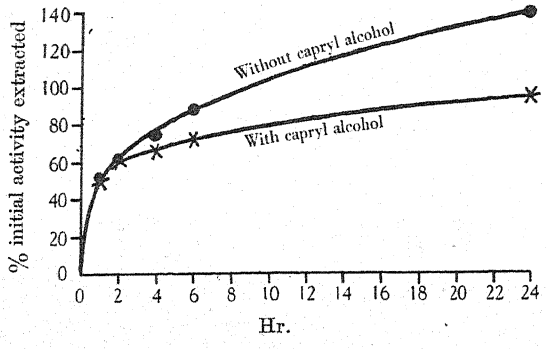

Fig. 3. Rate of extraction of enzyme from acetone powder at 37°. 25 mg. acetone-treated cells were suspended in 5 ml. water and incubated at 37°; to a similar suspension was added one drop of capryl alcohol, with shaking, and this also was incubated at 37°. Samples were withdrawn at intervals for the determination of decarboxylase activity in the extract.

have resulted from bacterial growth, and it is probable that it was present all the time but that its activity was somehow masked. Fig. 4 shows the variation of the total and of the extractable activity with the temperature of incubation. Heating the suspension to 70° for 30 min. almost doubled the

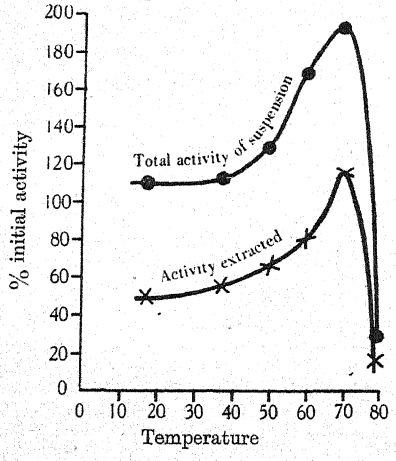

Fig. 4. Variation of total and extractable activity with temperature of incubation. Suspensions containing 60 mg. acetone powder in 2 ml. water were incubated at various temperatures for 30 min. and the decarboxylase activity of the extract was then determined.

total activity of the suspension, although at no temperature is more than about half the total activity extractable. This increase in activity cannot be due to production of new enzyme but must be due to freeing of enzyme previously masked. Probably the enzyme is present in the cell in two fractions, one of which is easily extracted and the other fixed to insoluble constituents of the cell from which it can be liberated only with difficulty.

At 70° (Fig. 5), as at 37°, much activity is extracted very rapidly; for 1 hr. thereafter the rate of extraction falls, and later destruction of the

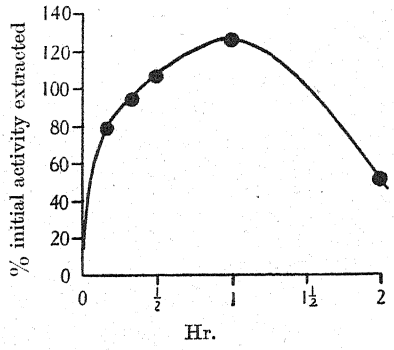

Fig. 5. Rate of extraction of enzyme at 70°. A suspension of 120 mg. of acetone powder in 4 ml. water was incubated at 70° and samples were withdrawn at intervals for the determination of decarboxylase activity in the extract.

enzyme sets in. The stability at 70° varies with different acetone powders. Thus one powder lost 69% of its activity after 1 hr. at 70°. The ease of extractability of the enzyme also varies (Table 2) for unknown reasons.

Table 2. *Variation in extractability of the enzyme*

The various acetone powders were incubated at 37° in aqueous suspension at a concentration of 5 mg./ml. for the times shown in the table. After incubation the suspensions were centrifuged and the decarboxylating activity of a suitable aliquot of the supernatant determined.

	Acetone powder preparation							
	A	B		C		D*	E*	F*
Incubation time (hr.) ...	1	1	4	1	4	22	24	24
Initial activity extracted (%)	62	13	52	43	80	284	22	76
Initial Q_{CO_2}	136	2300		2230		1065	1030	1210

* These suspensions contained capryl alcohol.

The amount of enzyme extracted is independent of the concentration of the acetone powder suspension within the range 1–30 mg./ml. With high concentrations, however, the centrifuging of residual insoluble material becomes difficult. A concentration of 15 mg. acetone powder/ml. seems to be the most suitable.

Purification of the enzyme

Adsorption on to alumina Cγ. The first step in the purification was adsorption on to alumina Cγ. Of the other adsorbing agents tried, kaolin adsorbed well at *p*H 4·0, and to some extent (30 %), at *p*H 5·0, but not at reactions alkaline to this; Ca phosphate adsorbed 84 % of the enzyme at *p*H 7·2 while kieselguhr adsorbed 78 % at *p*H 4·0. Alumina Cγ suspension (13–17 mg. alumina/ml.) possesses several advantages; at *p*H 5·0 and 6·0, it adsorbs 70–80 % of the enzyme, but at *p*H 7·0 and above less than 6 %. For adsorption, the extract was therefore brought to *p*H 5·0 by the addition of $\frac{1}{20}$ vol. of 2*M* acetate buffer. An extract derived from an acetone powder suspension of 5 mg./ml. requires $\frac{1}{10}$ vol. alumina suspension, but with stronger extracts more alumina is required, 0·3 vol. being sufficient up to 30 mg./ml.

Adsorption is very rapid though not instantaneous; 60 % is adsorbed in less than 1 min., 89 % in 5 min. and 93 % in 10 min. The rate of elution of the enzyme is very similar. 10 min. was allowed for both processes.

Elution of the enzyme. The alumina was suspended in *M*/5 phosphate buffer at *p*H 7·0 and after 10 min. was centrifuged. For maximum elution the volume of eluent should be at least double the volume of alumina suspension used in the adsorption of the enzyme.

Precipitation by ammonium sulphate. Solid $(NH_4)_2SO_4$ was now added to the clear yellow eluate to give 80 % saturation. The solid was used to keep the volume low: 75 g./100 ml. *of solvent* represents saturation, and other figures are based proportionally. The precipitate was separated by centrifuging and redissolved in a volume of distilled water equal to about 25 % of that of the eluate. Fractional precipitation with $(NH_4)_2SO_4$ failed to separate out the enzyme consistently in any one fraction probably owing to variations in protein impurities. Accordingly it was decided to remove more of the inactive protein before attempting fractional precipitation with $(NH_4)_2SO_4$.

Removal of inactive protein. The addition of $\frac{1}{20}$ vol. of 2*M* acetate buffer at *p*H 4·0 to the solution of the fraction precipitated by 80 % sat. $(NH_4)_2SO_4$ was found to precipitate much of the inactive material; subsequent addition with rapid mixing of $\frac{1}{5}$ vol. of ethanol to the same solution precipitated a further quantity. More ethanol ($\frac{1}{2}$ vol.), however, inactivates the enzyme.

The enzyme could be recovered from the alcoholic solution by adsorption on to alumina Cγ and elution with *M*/5 phosphate buffer of *p*H 7·0, using 0·3 vol. of alumina suspension and a volume of phosphate equal to the volume of the preparation before ethanol treatment. For the adsorption with alumina the *p*H remained at 4·0.

Fractional precipitation with ammonium sulphate. After the above treatment it was found that most of the enzyme was consistently precipitated in the 50–60 % sat. $(NH_4)_2SO_4$ fraction, and that this was also the purest fraction.

It should be pointed out that the degree of purification obtained in each stage varies considerably from one preparation to another but at the last stage the activity is never less than $Q^{C}_{CO_2} = 200{,}000$.

To carry purification further much time and starting material would be needed. From 6 to 9 g. of acetone-treated cells, which is the entire crop from 18 l. of medium, the total amount of protein in the enzyme preparation when an activity of 300,000 is reached, is of the order of 2–5 mg., of which 0·5–1·0 mg. is required for a reliable estimation of the C content. A few further treatments were, however, tried with variable results. Heating the preparation to 55° or even to 65° did not remove any inactive material; a second adsorption of the enzyme on to alumina Cγ and elution with *M*/5 phosphate did not increase activity; concentration of the final preparation, and repetition of the ethanol precipitation at *p*H 4·0, resulted, in one case, in division of the enzyme between the ethanol precipitate and the solution when the precipitate had a slightly increased activity, but in another case most of the activity was lost.

Difficulties in centrifuging the enzyme precipitates. Satisfactory sedimentation of the enzyme at high concentrations of $(NH_4)_2SO_4$ (60 % and above) presented difficulty. This was especially marked with the more highly purified preparations, and when a $Q^{C}_{CO_2}$ of 200,000–300,000 is reached the precipitate formed on 60 % saturation with $(NH_4)_2SO_4$ cannot be separated on the centrifuge at 3500 r.p.m. Such precipitates have to be filtered off through a G4 glass filter, a very slow process. The addition of a drop of capryl alcohol, followed by the thorough removal of dissolved air by evacuation, improves the sedimentation of the precipitates in the earlier stages of the purification, but fails in the final stage.

The final purification procedure. The details of one typical experiment on the purification of the enzyme are set out below:

(1) 5·2 g. of acetone-treated cells (E.U. = 4260; $Q^{C}_{CO_2} = 2810$) were suspended in about 1 l. of distilled water and incubated at 37° for 4 hr. The suspension was centrifuged and 1030 ml. of a cloudy yellow extract were obtained (E.U. = 1725; $Q^{C}_{CO_2} = 1180$).

(2) To the extract were added $\frac{1}{20}$ vol. of 2*M* acetate buffer of *p*H 5·0 and $\frac{1}{10}$ vol. of alumina Cγ suspension (16·7 mg. alumina/ml.). After 10 min. the alumina was spun off and suspended in 203 ml. *M*/5 phosphate buffer of *p*H 7·0. After another

10 min. this was centrifuged and 203 ml. of a clear yellow solution obtained (E.U. = 900; $Q^{C}_{CO_2} = 7040$).

(3) To the eluate were added 120 g. $(NH_4)_2SO_4$ giving 80 % saturation; the centrifuged precipitate gave a yellow solution in 37·5 ml. distilled water (E.U. = 710; $Q^{C}_{CO_2} = 45{,}000$).

(4) To this solution were added $\frac{1}{20}$ vol. 2*M* acetate buffer at *p*H 4·0 and then $\frac{1}{5}$ vol. ethanol, with vigorous shaking. After a few minutes the precipitate was spun off and 11 ml. of alumina Cγ suspension (strength as above) were added to the centrifugate; after 10 min. the alumina was spun off and the enzyme eluted for 10 min. with 37·5 ml. *M*/5 phosphate buffer at *p*H 7·0. A clear, slightly yellow solution was obtained (E.U. = 539; $Q^{C}_{CO_2} =$ 85,000).

(5) To this eluate were added 13·6 g. $(NH_4)_2SO_4$ giving 50 % saturation; the precipitate was spun off and discarded. The centrifugate contained the enzyme (E.U. = 440; $Q^{C}_{CO_2} = 172{,}000$).

(6) To the centrifugate 2·73 g. $(NH_4)_2SO_4$ were added giving 60 % saturation. The precipitate was filtered off and dissolved in 5·2 ml. distilled water; the solution contained 181 units of $Q^{C}_{CO_2} = 330{,}000$. This was the final preparation (Table 3). The most active preparation so far obtained had an activity of 404,000 (but see below on the dissociation of the enzyme).

Table 3. *Purification of the enzyme*

Treatment	Enzyme units	$Q^{C}_{CO_2}$
(1) Original acetone powder	4260	2,810
(2) Aqueous extract	1725	1,180
(3) Adsorption on to alumina and elution with *M*/5 phosphate *p*H 7·0	900	7,040
(4) Precipitation by 80 % saturation with $(NH_4)_2SO_4$	710	45,000
(5) Precipitation by $\frac{1}{5}$ vol. ethanol at *p*H 4·0 followed by adsorption on to alumina and elution with phosphate	539	85,000
(6) Precipitation by $(NH_4)_2SO_4$ between the limits 50–60 % saturation	181	330,000

Properties of the purified enzyme

Strong solutions of the purest enzyme yet obtained are very slightly yellow, perhaps owing to impurities, since yellow pigments are eliminated at almost every stage of the purification. The ultra-violet absorption spectrum determined in the Spekker spectrophotometer shows a typical protein absorption curve having a maximum at 275mμ and log I_0/I of 2·8 at this wave-length for a solution containing 0·533 mg. C/ml. (about 1 mg. protein). All the following experiments were carried out with the enzyme preparation of $Q^{C}_{CO_2} = 404{,}000$.

(1) *Reaction velocity: effect of* p*H*. This was determined in *M*/5 acetate buffer. The optimum *p*H is seen to lie at 4·9–5·0 (Fig. 6).

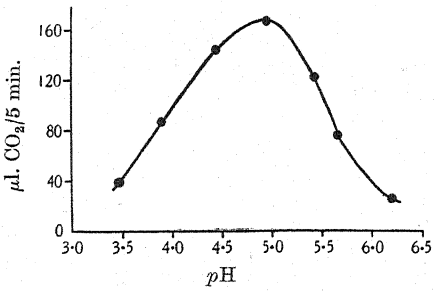

Fig. 6. Effect of *p*H on the rate of decarboxylation by the purified enzyme. Manometers contained 0·5 ml. enzyme solution, 0·3 ml. 2*M* acetate buffer of the required *p*H, 0·5 ml. *M*/2 Na acetoacetate; total vol. 3·0 ml.; temp. 37·5°.

(2) *Effect of substrate concentration.* Fig. 7 shows that the affinity of the enzyme for its substrate is fairly low, a concentration of 0·05*M* acetoacetic acid being required to saturate the enzyme, the half-speed concentration being 0·008*M*.

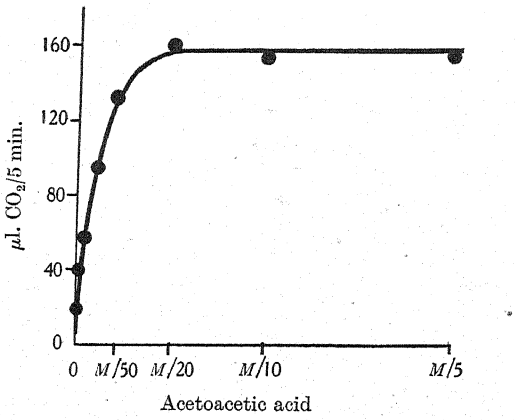

Fig. 7. Effect of substrate concentration on the rate of decarboxylation by the purified enzyme. Manometers contained 0·5 ml. enzyme solution, 0·3 ml. 2*M* acetate buffer *p*H 5·0, *M*/2 Na acetoacetate to give the required concentration; total vol. 3·0 ml.; temp. 37·5°.

(3) *Effect of temperature.* The temperature coefficient increases slightly with rise in temperature (Fig. 8). For the range 20–30° the $Q_{10°} = 1{\cdot}82$ and for 30–40° the $Q_{10°} = 2{\cdot}16$.

Fig. 9 shows the effect of 5 min. exposure to various temperatures, the activity being determined at 37·5°. Little destruction occurs up to 70°; above 70° the enzyme is rapidly inactivated, being almost completely destroyed in 5 min. at 85°. Inactivation

at 60° occurs at a fairly steady rate (Fig. 10). There was no detectable loss in activity of an aqueous solution that had been stored for 5 weeks in the ice-chest at 4°.

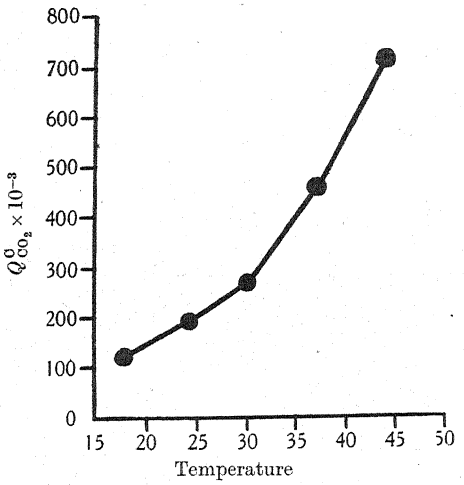

Fig. 8. Effect of temperature on the rate of decarboxylation by the purified enzyme. Manometers contained 0·5 ml. enzyme solution, 0·3 ml. 2 *M* acetate buffer *p*H 5·0, 0·5 ml. *M*/2 Na acetoacetate; total vol. 3·0 ml.; temp. as indicated.

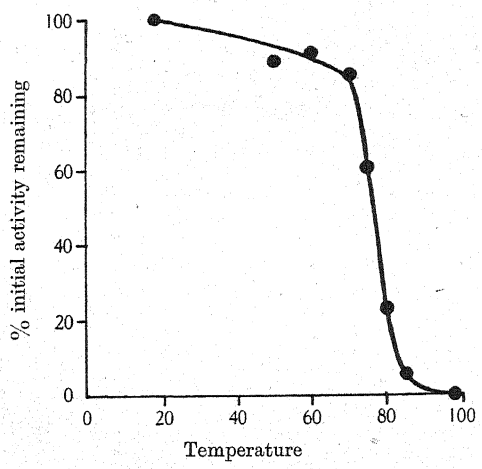

Fig. 9. Effect of temperature on the stability of the enzyme. 1 ml. enzyme preparation was heated for 5 min. at the temperatures shown in the figure; 0·5 ml. was then used for the determination of activity under the optimum conditions.

(4) *Effect of inhibitors.* The effects of well-known enzyme inhibitors are given in Table 4. Unlike yeast carboxylase [Green, Herbert & Subrahmanyan, 1941] the enzyme is comparatively insensitive to Cu, though very sensitive to Ag and Hg; KCN is a fairly powerful inhibitor of it and NaN_3 inhibits it in high concentrations. α-Keto-acids inhibit it to

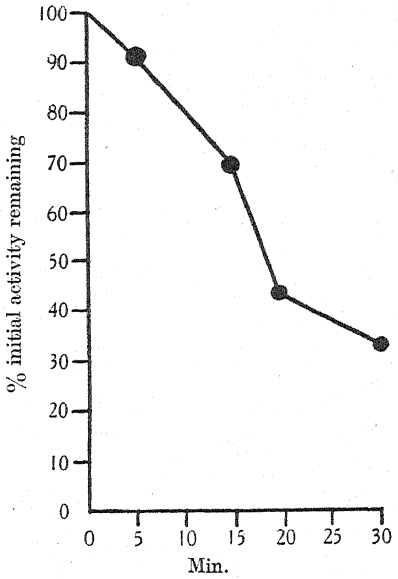

Fig. 10. Effect of time on the activity of the enzyme incubated at 60°. 4 ml. of the enzyme preparation were incubated at 60° and samples (0·5 ml.) were withdrawn at intervals for determination of decarboxylase activity.

varying degrees and outstanding in this class is acetopyruvic acid which causes 65% inhibition at a concentration of $M \times 10^{-6}$. The structural relationships between this substance and acetoacetic

Table 4. *Effect of inhibitors on decarboxylase activity*

Inhibitor	Conc. *M*	Inhibition %
Heavy metals:		
$FeCl_3$	10^{-2}	45
$CuSO_4$	2×10^{-2}	41
$AgNO_3$	10^{-5}	90
$HgCl_2$	10^{-5}	100
General:		
Capryl alcohol	Satd. sol.	0
Na_2S	10^{-1}	0
Urethane	10^{-1}	16
Toluene	Satd. sol.	47
NaN_3	10^{-2}	53
Iodoacetic acid	10^{-2}	63
KCN	10^{-4}	82
Competitive:		
Acetone	1	10
Methyl ethyl ketone	1	17
Acetone dicarboxylic acid	5×10^{-2}	22
Pyruvic acid	5×10^{-2}	64
α-Ketoglutaric acid	5×10^{-2}	70
Ethyl acetoacetate	10^{-1}	72
Malonic acid	10^{-1}	76
Oxaloacetic acid	10^{-2}	44
Acetopyruvic acid	10^{-6}	65

acid suggest that the inhibition is of a competitive kind, acetopyruvic acid being the most closely related and also the most powerfully inhibitory. The marked inhibition of acetoacetic acid decarboxylase by low concentrations of acetopyruvic acid disposes entirely of the theory that this substance is an intermediate in the formation of acetone.

(5) *Specificity of the enzyme.* Table 5 shows that the enzyme is specific for the decarboxylation of acetoacetic acid within the range of substances tested.

Table 5. *Specificity of the enzyme*

Substrate	Conc. *M*	CO_2 μl./5 min.
Acetoacetic acid	0·08	138
Ethyl acetoacetate	0·1	0
Pyruvic acid	0·05	0
Acetopyruvic acid	0·05	0
Oxaloacetic acid	0·01	0
α-Ketoglutaric acid	0·05	0
Acetone dicarboxylic acid	0·05	0

All the determinations were made in *M*/5 acetate *p*H 5·0.

(6) *Effect of enzyme concentration.* Fig. 11 (curve 1) shows that the reaction velocity increases more rapidly with increasing enzyme concentration than would be expected on the basis of a linear

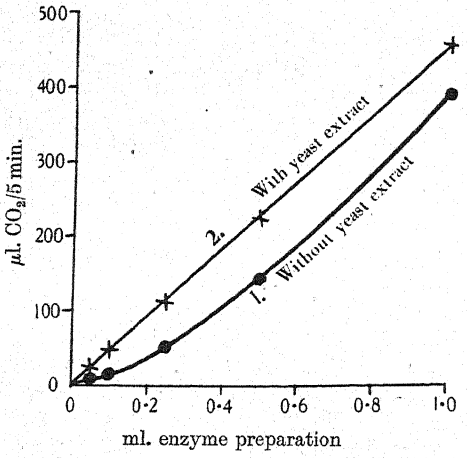

Fig. 11. Effect of enzyme concentration on the rate of decarboxylation. (1) without yeast extract, (2) in presence of yeast extract. Manometers contained 0·3 ml. 2*M* acetate buffer *p*H 5·0, 0·5 ml. *M*/2 Na acetoacetate, 0·5 ml. of a boiling-water extract of dried brewer's yeast in one series, and enzyme solution as shown in the figure; total vol. 3·0 ml.; temp. 37·5°.

relationship. This suggests that at low concentrations the enzyme may dissociate into a specific protein and a coenzyme, only the undissociated enzyme being catalytically active. That this is the case is shown by curve 2 where the reaction velocity has been determined in the presence of a boiling-water extract of dried brewer's yeast; here the relationship between enzyme concentration and reaction velocity is linear. Presumably the extract contained the coenzyme in sufficient concentration to counteract the dissociation of the enzyme.

The concentration of enzyme in the region where dissociation is greatest is only about 0·7 μg./ml. and it is in fact only because of the high activity of the enzyme that the dissociation can be observed. Assuming that the enzyme is pure it can be calculated from the curves of Fig. 11 that the dissociation constant for the enzyme is very approximately $1{\cdot}55 \times 10^{-8}$ mol./l. This may be compared with the value of $2{\cdot}5 \times 10^{-7}$ mol./l. for *d*-amino-acid oxidase [Warburg & Christian, 1938*a*].

Attempts to separate enzyme into protein and coenzyme

The dissociation of the enzyme in low concentrations should make it possible to separate the protein from the coenzyme by dialysis; all attempts so far, however, have been unsuccessful; dialysis for 24 hr. at 37° of 30 ml. of a solution containing about 1 μg. enzyme/ml. against 4 l. of *M*/5 acetate buffer at *p*H 5·0 effected no change in the activity of the enzyme, these conditions being precisely those under which dissociation is observed in the manometer.

Precipitation with ammoniacal $(NH_4)_2SO_4$, which reversibly dissociates yeast carboxylase [Green *et al.* 1941] does not so dissociate acetoacetic acid decarboxylase. At room temperature, at 45° for 5 min., at 55° for 5 min. and at 0° for 20–40 hr., there is a varying loss of activity, but in no case can the activity be restored by adding a boiled suspension of acetone powder or of dried yeast. The same is true after precipitation by $(NH_4)_2SO_4$ in acid solution by the method of Warburg & Christian [1938*b*] as described by Weil-Malherbe [1939], while increase in the final acid concentration up to *M*/22 HCl results in a 50 % loss of activity which also cannot be restored. Adjustment of the enzyme solution to *p*H 2 or *p*H 3 followed by precipitation with 10 vol. acetone results in complete loss of activity. Finally, 2 ml. of enzyme solution were dialysed for 22 hr. at 17° against 800 ml. of a solution containing 9 parts 70 % sat. $(NH_4)_2SO_4$ solution and 1 part conc. NH_3 solution, and this was followed by dialysis for 1 hr. against running tap water. The enzyme was then found to have lost 76 % of its activity, which loss could not be restored by adding boiled cells. It must be assumed in view of these failures to restore the activity of the enzyme that all the above procedures have not caused the enzyme to dissociate but have merely inactivated it.

The nature of the coenzyme

Despite the failure to split the enzyme reversibly into a coenzyme and a specific protein, some information as to the identity of the coenzyme has been obtained by studying the reversal of the dissociation that occurs at low enzyme concentrations. As has been shown, yeast extract and an extract of acetone-treated cells can counteract the dissociation, hence the ability of other substances to replace the extracts was investigated. The compounds tested were cocarboxylase, thiamin monophosphate, thiamin, muscle adenylic acid, cozymase (60–70 % pure), an old preparation of diaphorase containing a considerable proportion as free flavin and, finally, riboflavin had already been shown to be inactive with fair certainty. There remained the first three possibilities. Table 7 shows the effect of boiling, and of hydrolysis in acid and alkali, on the activity of the diaphorase preparation. It is seen that boiling and acid hydrolysis do not alter the activity, but that hydrolysis with alkali causes some inactivation. The first result shows that diaphorase itself cannot be the coenzyme, while the result of acid hydrolysis rules out riboflavin adenine dinucleotide since, under the conditions used, this substance would be hydrolysed into adenylic acid and riboflavin phosphate [Abraham, 1939]. Riboflavin phosphate, however, is not split under these conditions [Kühn & Rudy, 1936]. Unfortunately it has been impossible to complete these experiments at the present time, but it seems possible that riboflavin phosphate is actually the coenzyme; the effect of hydrolysis with alkali is evidence in support of this idea.

Table 6. *Inhibition of dissociation by various substances*

The manometers contained 0·3 ml. 2*M* acetate buffer *p*H 5·0, 0·5 ml. *M*/2 acetoacetic acid, 0·1 ml. enzyme solution containing about 0·7 μg. protein; the total vol. was 3·0 ml. Temp. 37·5°. The diaphorase preparation contained total flavin equivalent to about 20 μg. lactoflavin/ml.

	Substance tested												
	Nil	10 % yeast extract 0·5 ml.	1 % extract of acetone-treated cells 0·5 ml.	Co-carboxylase 50 μg.	Thiamin monophosphate 40 μg.	Thiamin 100 μg.	Muscle adenylic acid 100 μg.	Co-zymase 100 μg.	Lactoflavin 61 μg.	Lactoflavin 6 μg.	Diaphorase prep. 0·1 ml.	Diaphorase prep. 0·01 ml.	Diaphorase prep. 0·001 ml.
μl. CO_2/5 min.	18	54	53	21	15	22	23	43	38	19	51	48	30

riboflavin. The results (Table 6) show that adenylic acid and the thiamin derivatives are totally inactive, while cozymase and riboflavin exhibit submaximal activity; the diaphorase preparation on the other hand is maximally active at a concentration of 0·0033 ml./ml. (about 0·07 μg. riboflavin/ml.). It is probable that the activity of riboflavin and of the cozymase preparation is due to an impurity, since even large amounts of these substances do not show maximum activity.

The active diaphorase preparation was originally about 90 % pure. There were therefore five possibilities, that the active substance was (*a*) diaphorase itself, (*b*) the prosthetic group of diaphorase, riboflavin adenine dinucleotide, (*c*) riboflavin phosphate, (*d*) riboflavin, and (*e*) an impurity in the diaphorase preparation. In view of the small amount of the diaphorase preparation required for maximum activity the last possibility did not seem likely, although it has not yet been excluded, while

Table 7. *Effect of boiling and of hydrolysis by acid and alkali on the activity of the diaphorase preparation*

The manometers contained the equivalent of 0·1 ml. of the diaphorase preparation; the other contents were as described in Table 6.

Treatment of diaphorase ...	None	Heated at 100° for 5 min.	Heated at 96° for 60 min. in *N*/20 HCl	Boiled for ½ min. in *N*/10 NaOH	Control, no diaphorase
μl. CO_2/5 min.	52	50	46	36	18

SUMMARY

1. The purification and properties of acetoacetic acid decarboxylase of *Cl. acetobutylicum* have been described.

2. The purified enzyme is quite stable at ordinary temperatures; it is optimally active at *p*H 5·0 and is specific for acetoacetic acid.

3. In dilute solution the enzyme is dissociated but it has not been possible to separate the component parts.

4. The dissociation can be prevented by a preparation of diaphorase at a concentration equivalent to 0·07 μg. riboflavin/ml. Riboflavin is inactive. Mild acid hydrolysis (*N*/20 HCl for 60 min. at 96°) does not affect this activity of the diaphorase preparation.

5. It is tentatively suggested that riboflavin phosphate may be the prosthetic group of the enzyme.

I wish to express my sincere thanks to Dr M. Stephenson for her constant encouragement and advice throughout this work; to Miss H. Epps for kindly carrying out the microcarbon determinations; to Drs M. Dixon, E. F. Gale, R. Hill and Prof. Friedmann for much valuable advice; and to Dr Dixon and Dr Hartree for gifts of various nucleotides. I also wish to express my gratitude and thanks to the Medical Research Council for a full-time grant.

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The Influence of Dietary Fat of Varying Unsaturation on the Component Acids of Cow Milk Fats

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In feeding trials in which the basal ration of a group of dairy cows was supplemented by 1 lb./day of various fatty oils, Brown, Dustman & Weakley [1941] observed that the iodine value (i.v.) of the milk fat was altered in different ways by different fats, a feeding period of about 3 weeks being necessary before the extent of the change in i.v. reached its maximum. Coconut oil caused a slight reduction of 4 or 5 units in the i.v. of the milk fat, both crude and refined soya bean oil (i.v. *ca.* 127) increased it greatly (by over 50 %), and hydrogenated soya bean oil (i.v. 17) increased it slightly (by about 5 or 6 units), as well as having a pronounced laxative action on the cows. These authors ascribed the slight increase in the unsaturation caused by feeding the hydrogenated oil to desaturation of the fat in the ration, an assumption for which there seems to be no evidence.

Similar alterations in the unsaturation of cow milk fats (as indicated by their i.v.'s alone), which result from administration of specific fats in the diet, have been published. Thus, Holland & Buckley [1918; 1923] who fed cows on a basal ration, and on the basal ration supplemented by $\frac{3}{4}$ lb. daily of coconut, groundnut, maize or soya bean oils, observed a slight fall in i.v. with the coconut oil diet, but marked increases (35–45 %) with the three liquid fats. Again, Hill & Palmer [1938] found that the inclusion of maize, cottonseed or linseed oils in the diet increased the i.v.'s, and slightly diminished the Reichert-Meissl values of the corresponding milk fats; tallow slightly increased the milk fat i.v., while butter slightly decreased it, and coconut oil decreased it by 4 or 5 units. Such observations clearly only measure changes in the mean unsaturation of the milk fats, and do not indicate which of the component acids has significantly altered in amount. Thus, whilst oleic acid is the chief unsaturated acid, at least six others may be present in small proportions in cow milk fat, together with nine saturated acids (of which palmitic acid is the chief, though there are appreciable amounts of butyric, myristic and stearic acids). For a clear picture of milk-fat component acids a detailed analysis of the proportions of each acid is necessary.

Few detailed examinations have been made of the effect of supplementary fats in the cow's diet. Hilditch & Sleightholme [1930] examined milk fats from cows receiving coconut cake or soya bean cake in addition to their normal winter ration; the daily ration of cake (7 lb. coconut, 5 lb. soya bean) could not have contained more (and possibly much less) than 6–7 oz. of fatty oil. The milk fat from cows receiving coconut cake contained less oleic and more lauric and myristic acids than normal, whilst that from animals receiving soya bean cake had somewhat less oleic but the normal amount of octadecadienoic acid, and was otherwise similar to the control milk fats. Hilditch & Thompson [1936] observed similar effects when 4 oz. of linseed, rape or cod-liver oil were added to the daily basal ration of cows. With linseed or rape oils, the component acids differed little, but the proportion of palmitic acid was reduced and that of oleic somewhat increased; with linseed oil, no linolenic acid was present, but the proportion (5·9 % by wt.) of octadecadienoic acids was higher than usual (*ca.* 3 %), whilst with rape oil the octadecadienoic acids were normal (3·3 %) but there was a similar amount of erucic acid. With cod-liver oil the results were more striking: the milk fat here contained an unusually large amount (5·8 %) of highly unsaturated acids

of the C_{20} and C_{22} series, its content of oleic acid was much higher, and the typical lower saturated acids of cow milk fat (notably butyric and hexanoic) were reduced by half. On the hypothesis that milk fat is produced in the mammary gland from blood glycerides by conversion of some of the oleo-glycerides to butyro- and other glycerides by an oxidation-reduction system, the results observed with cod-liver oil might be due to partial suppression of the normal metabolic processes whereby the mammary gland converts blood glycerides into milk glycerides, owing to absorption of some of the highly unsaturated fish oil glycerides.

Smith & Dastur [1938] showed that a similar reduction in the amounts of lower saturated acids (from butyric to myristic) and in tetra- and hexadecenoic acids, with a large increase in the proportion of oleic acid, occurred in milk fat from cows maintained at a very low dietary level, the effect of fasting being apparently to upset the normal metabolic processes of milk fat production.

A series of experiments somewhat similar to those of Brown *et al.* [1941] was, therefore, made in order to review the effect of supplementing a basal diet with fats at different levels of unsaturation, and, also, to ascertain whether desaturation of saturated ingested fats occurs as postulated by these workers. Hence, groups of cows were fed on a basal ration either alone or supplemented by refined groundnut oil (i.v. 88), hydrogenated groundnut oil (i.v. 45 and i.v. 17), or palm-kernel oil (i.v. 17). Thus the fatty oil supplements were (1) a natural fatty oil rich in oleic and linoleic acids (about 55 and 20 % of the total fatty acids respectively), (2) a hydrogenated oil of a similar degree of mean unsaturation to that of a soft butterfat, (3) a much more saturated hydrogenated groundnut oil; and, for comparison with the latter, (4) a natural vegetable fat (palm-kernel oil) of the same mean unsaturation but entirely different fatty oil composition, consistency and melting-point.

METHODS

The 15 Ayrshire heifers used had calved in Dec. 1941 and Jan. 1942 and belonged to Messrs J. Bibby and Sons, Ltd., Home Farm, Puddington, Wirral, where the experimental feeding, milk collection, and butter-making were carried out in February and March of 1942 under the supervision of Mr A. H. Blissett. The cows, which were at approximately the same stage of lactation, were divided into five groups of three, and were fed as follows:

Group I. Basal diet: 21 lb. medium hay, 14 lb. swedes, 6 lb. oats and 2 lb. groundnut meal per day.

Group II. Basal diet plus 8 oz. refined groundnut oil (i.v. 88) daily.

Group III. Basal diet plus 8 oz. hydrogenated groundnut oil (i.v. 45) daily.

Group IV. Basal diet plus 8 oz. hydrogenated groundnut oil (i.v. 17) daily.

Group V. Basal diet plus 8 oz. refined palm-kernel oil (i.v. 17) daily.

Only half the fat ration used by Brown *et al.* [1941] was given in order if possible to avoid undue laxative action. Very slight laxative action was observed only in group IV; the other groups were normal throughout the experimental period of about 35 days. During the first 3 days the oil supplements were increased gradually till the full daily ration of 8 oz. was being given. The i.v.'s of the milk fats were observed at intervals of a few days: during the first 2–3 weeks the values were variable but thereafter became more constant and, after the 34th day, 2 days' supply of milk was collected and converted into butter for detailed analysis. There was no significant alteration in the milk yields, nor in the proportions of fat and non-fat solids in the milk, in any of the groups during the period of the experiment. The general analytical characteristics of the milk fats from these butters are given in Table 1.

Table 1. *General analytical characteristics of milk fats*

Group ...	I	II	III	IV	V
Fat supplement	Control	G.N.O. (i.v. 88)	H.G.N.O. (i.v. 45)	H.G.N.O. (i.v. 17)	P.K.O. (i.v. 17)
Iodine value	39·2	43·8	43·3	38·8	38·0
Reichert-Meissl value	30·0	23·4	23·6	28·2	25·0
Polenske value	1·5	0·9	1·0	1·5	1·6
Kirschner value	23·5	19·4	19·0	23·1	21·6
Sap. equiv.	248·0	258·8	259·8	251·7	250·5

Alterations in milk fat i.v. following the inclusion of the fatty supplements in the diet were less marked than in some of the earlier investigations, including those of Brown *et al.* [1941]. This may be partly due to the smaller amount of fat given, which was only half of that used by Brown *et al.*, but is probably due to the fact that the i.v. of the milk fat of cows fed on the basal diet alone was much higher than in these other studies. Nevertheless, the i.v.'s of milk fats from cows receiving either refined groundnut oil or hydrogenated groundnut oil (i.v. 45) were definitely higher than that of the control, while the i.v. of milk fat from cows receiving palm-kernel oil was slightly lower than that of the control. There was no significant difference between the i.v. of the milk fat of cows which had received the hydrogenated groundnut oil (i.v. 17) and that of the control.

The component acids in the five milk fats were determined by separation and ester-fractionation as described by Hilditch & Jasperson [1941], the

fatty acids from 400 g. of each specimen being used for analysis (Table 2).

The percentages (wt. and mol.) of the component acids of the five milk fats are arranged for comparison in Table 3 (p. 241).

Table 2. *Component acids of cow milk fats*

	Steam-volatile acids	Non-steam-volatile 'Solid' acids	Non-steam-volatile 'Liquid' acids	Total	% wt.	% mol.
Group I. Cows fed on basal diet only						
% of total fat ...	(7·2)	(42·4)	(50·4)			
Butyric	3·94	—	—	3·94	4·0	10·5
Hexanoic	2·27	—	—	2·27	2·3	4·6
Octanoic	0·66	—	0·16	0·82	0·8	1·3
Decanoic	0·18	—	1·76	1·94	1·9	2·7
Lauric	—	0·13	2·06	2·19	2·2	2·6
Myristic	—	4·89	4·43	9·32	9·3	9·6
Palmitic	—	21·77	3·62	25·39	25·5	23·4
Stearic	—	11·76	—	11·76	11·8	9·7
As arachidic	—	0·83	—	0·83	0·8	0·6
Decenoic	—	—	0·19	0·19	0·2	0·3
Dodecenoic	—	—	0·21	0·21	0·2	0·2
Tetradecenoic	—	0·04	0·88	0·92	0·9	1·0
Hexadecenoic	—	0·47	1·81	2·28	2·3	2·1
Oleic	0·15	2·49	31·56	34·20	34·3	28·6
Octadecadienoic	—	—	2·13	2·13	2·1	1·8
Unsatd. C_{20-22}	—	—	1·38	1·38	1·4	1·0
Unsaponifiable	—	0·02	0·21	0·23	—	—
Group II. Cows fed on basal diet +8 oz. of groundnut oil (i.v. 88) daily						
% of total fat ...	(5·3)	(42·4)	(52·3)			
Butyric	2·93	—	—	2·93	2·9	8·2
Hexanoic	1·49	—	—	1·49	1·5	3·2
Octanoic	0·38	—	0·08	0·46	0·5	0·8
Decanoic	0·37	—	0·98	1·35	1·4	1·9
Lauric	—	0·10	1·63	1·73	1·7	2·1
Myristic	—	2·91	4·37	7·28	7·3	7·9
Palmitic	—	21·20	2·47	23·67	23·8	22·8
Stearic	—	12·61	—	12·61	12·7	10·9
As arachidic	—	1·22	—	1·22	1·2	1·0
Decenoic	—	—	0·10	0·10	0·1	0·1
Dodecenoic	—	—	0·15	0·15	0·2	0·2
Tetradecenoic	—	0·03	0·70	0·73	0·7	0·8
Hexadecenoic	—	0·61	1·54	2·15	2·1	2·1
Oleic	0·13	3·66	36·64	40·43	40·6	35·2
Octadecadienoic	—	—	1·82	1·82	1·8	1·6
Unsatd. C_{20-22}	—	—	1·53	1·53	1·5	1·2
Unsaponifiable	—	0·06	0·29	0·35	—	—
Group III. Cows fed on basal diet +8 oz. of hydrogenated groundnut oil (i.v. 45) daily						
% of total fat ...	(5·3)	(43·9)	(50·8)			
Butyric	2·72	—	—	2·72	2·7	7·6
Hexanoic	1·54	—	—	1·54	1·5	3·3
Octanoic	0·40	—	0·15	0·55	0·6	0·9
Decanoic	0·51	—	0·97	1·48	1·5	2·1
Lauric	—	0·35	1·61	1·96	2·0	2·4
Myristic	—	2·76	3·38	6·14	6·2	6·7
Palmitic	—	18·89	2·87	21·76	21·8	21·0
Stearic	—	16·53	—	16·53	16·6	14·4
As arachidic	—	1·04	—	1·04	1·0	0·8
Decenoic	—	—	0·09	0·09	0·1	0·1
Dodecenoic	—	0·01	0·17	0·18	0·2	0·2
Tetradecenoic	—	0·03	0·55	0·58	0·6	0·6
Hexadecenoic	—	0·66	1·59	2·25	2·3	2·2
Oleic	0·13	3·60	35·26	38·99	39·0	34·4
Octadecadienoic	—	—	3·28	3·28	3·3	2·9
Unsatd. C_{20-22}	—	—	0·59	0·59	0·6	0·4
Unsaponifiable	—	0·03	0·29	0·32	—	—

Table 2 (*cont.*)

	Steam-volatile acids	Non-steam-volatile 'Solid' acids	Non-steam-volatile 'Liquid' acids	Total	% wt.	% mol.
Group IV. Cows fed on basal diet +8 oz. of hydrogenated groundnut oil (i.v. 17) daily						
% of total fat ...	(6·9)	(43·7)	(49·4)			
Butyric	3·60	—	—	3·60	3·6	9·8
Hexanoic	2·08	—	—	2·08	2·1	4·3
Octanoic	1·00	—	0·16	1·16	1·2	1·9
Decanoic	0·04	—	1·48	1·52	1·5	2·1
Lauric	—	0·12	1·78	1·90	1·9	2·3
Myristic	—	4·13	4·04	8·17	8·2	8·5
Palmitic	—	21·15	3·65	24·80	24·9	23·2
Stearic	—	13·79	—	13·79	13·8	11·6
As arachidic	—	1·21	—	1·21	1·2	0·9
Decenoic	—	—	0·13	0·13	0·1	0·2
Dodecenoic	—	—	0·15	0·15	0·2	0·2
Tetradecenoic	—	0·04	0·73	0·77	0·8	0·8
Hexadecenoic	—	0·41	1·09	1·50	1·5	1·4
Oleic	0·18	2·83	32·50	35·51	35·6	30·0
Octadecadienoic	—	—	1·73	1·73	1·7	1·5
Unsatd. C_{20-22}	—	—	1·70	1·70	1·7	1·3
Unsaponifiable	—	0·02	0·26	0·28	—	—
Group V. Cows fed on basal diet +8 oz. of palm-kernel oil (i.v. 17) daily						
% of total fat ...	(6·1)	(42·2)	(51·7)			
Butyric	3·19	—	—	3·19	3·2	8·6
Hexanoic	1·67	—	—	1·67	1·7	3·4
Octanoic	1·00	—	0·14	1·14	1·2	1·9
Decanoic	0·08	—	1·24	1·32	1·3	1·8
Lauric	—	0·50	5·47	5·97	6·0	7·1
Myristic	—	6·11	4·76	10·87	10·9	11·3
Palmitic	—	19·26	2·87	22·13	22·2	20·6
Stearic	—	11·97	—	11·97	12·0	10·0
As arachidic	—	1·10	—	1·10	1·1	0·8
Decenoic	—	—	0·13	0·13	0·1	0·2
Dodecenoic	—	0·01	0·43	0·44	0·4	0·5
Tetradecenoic	—	0·04	1·00	1·04	1·0	1·1
Hexadecenoic	—	0·27	2·04	2·31	2·3	2·2
Oleic	0·16	2·86	30·29	33·31	33·5	28·1
Octadecadienoic	—	—	1·15	1·15	1·2	1·0
Unsatd. C_{20-22}	—	—	1·83	1·83	1·9	1·4
Unsaponifiable	—	0·08	0·35	0·43	—	—

DISCUSSION

The mean unsaturation of the five milk fats, as indicated by their i.v.'s (Table 1), shows parallelism, but not quantitative agreement, with their contents of oleic acid as determined in the ester-fractionation analyses. Similarly, the almost identical Reichert-Meissl and Kirschner values of milk fats I and IV, and also of milk fats II and III, are reflected in the contents of the lower saturated acids in these two pairs of milk fats as revealed by the detailed analyses. The data (Table 3) show that ingestion of groundnut oil, a fat richer in oleic glycerides than milk fat, is followed by a significant increase in oleic acid in the milk fat (group II), a slight fall in palmitic and a large fall in butyric-decanoic acids. On the other hand, though groundnut oil contains about 20% linoleic acid, the content of octadecadienoic acid is not appreciably altered; nor have the arachidic, behenic or lignoceric acids (about 6% of the groundnut oil), passed into the milk fat.

Table 3. *Component acids of the five milk fats*

Group ...	I	II	III	IV	V
Dietary fat supplement ...	Nil	G.N.O. (i.v. 88)	H.G.N.O. (i.v. 45)	H.G.N.O. (i.v. 17)	P.K.O. (i.v. 17)
Acid	Weight percentages				
Butyric	4·0	2·9	2·7	3·6	3·2
Hexanoic	2·3	1·5	1·5	2·1	1·7
Octanoic	0·8	0·5	0·6	1·2	1·2
Decanoic	1·9	1·4	1·5	1·5	1·3
Lauric	2·2	1·7	2·0	1·9	6·0
Myristic	9·3	7·3	6·2	8·2	10·9
Palmitic	25·5	23·8	21·8	24·9	22·2
Stearic	11·8	12·7	16·6	13·8	12·0
As arachidic	0·8	1·2	1·0	1·2	1·1
Decenoic	0·2	0·1	0·1	0·1	0·1
Dodecenoic	0·2	0·2	0·2	0·2	0·4
Tetradecenoic	0·9	0·7	0·6	0·8	1·0
Hexadecenoic	2·3	2·1	2·3	1·5	2·3
Oleic	34·3	40·6	39·0	35·6	33·5
Octadecadienoic	2·1	1·8	3·3	1·7	1·2
Unsatd. C_{20-22}	1·4	1·5	0·6	1·7	1·9
	Molar percentages				
Butyric	10·5	8·2	7·6	9·8	8·6
Hexanoic	4·6	3·2	3·3	4·3	3·4
Octanoic	1·3	0·8	0·9	1·9	1·9
Decanoic	2·7	1·9	2·1	2·1	1·8
Lauric	2·6	2·1	2·4	2·3	7·1
Myristic	9·6	7·9	6·7	8·5	11·3
Palmitic	23·4	22·8	21·0	23·2	20·6
Stearic	9·7	10·9	14·4	11·6	10·0
As arachidic	0·6	1·0	0·8	0·9	0·8
Decenoic	0·3	0·1	0·1	0·2	0·2
Dodecenoic	0·2	0·2	0·2	0·2	0·5
Tetradecenoic	1·0	0·8	0·6	0·8	1·1
Hexadecenoic	2·1	2·1	2·2	1·4	2·2
Oleic	28·6	35·2	34·4	30·0	28·1
Octadecadienoic	1·8	1·6	2·9	1·5	1·0
Unsatd. C_{20-22}	1·0	1·2	0·4	1·3	1·4

It has now been established [Graham, Jones & Kay, 1936; Maynard, McCay, Ellis, Hodson & Davis, 1938; Shaw & Petersen, 1938; Shaw, 1939] that milk glycerides are produced from glycerides in the blood which passes through the mammary gland; this complicates the interpretation of results, since the introduction of fatty acids, or modified fatty acids, from the ingested fats must be considered in terms of the mixed glycerides of the butter and not merely as individual fatty acids. 20–30 % of groundnut oil probably consists of glycerides in which, however, linoleic, arachidic and higher acids are not present [Hilditch & Meara, 1942]; they are palmitodiolein, stearodiolein or triolein. The effect on the milk fat of feeding groundnut oil might be due to admixture of some or all of these glycerides with normal milk fat, and also to interference with the mammary gland, whereby the production of the lower saturated glycerides normal to milk fat is restricted.

In group III the supplementary fat was groundnut oil which had been hydrogenated to give an i.v. of 45·5, i.e. approximately that of butterfat. It contained, however, about 52 % of oleic (and *iso*-oleic) acids and about 33 % of stearic acid; of the glycerides about 10 % were completely saturated, 65 % were monosaturated dioleins (or *iso*-oleins) and 25 % mono-oleo- (or *iso*-oleo-) disaturated glycerides. The effect on the milk fat of feeding this is broadly similar to that of groundnut oil itself, i.e. increase in oleic acid content, further diminution in palmitic acid and diminution in butyric-decanoic acids. The increase in the stearic acid content is consistent with the larger amount of this acid (as oleo-stearo-glycerides) in the hydrogenated oil.

Both in group II and group III ingestion of fat containing more combined oleic (or *iso*-oleic) acid than normal butterfat increased the oleic acid contents of the resulting milk fats. The similarity in the content of oleic acid in milk fats II and III may perhaps be due to the fact that the oleic (+*iso*-oleic) acid content of groundnut oil and hydrogenated groundnut oil (i.v. 45) are similar (52–56 %). This selective absorption of oleic (as distinct from linoleic) acid by the mammary gland may explain why [Cranfield, 1916] cows fed with groundnut cake give softer butters than those fed with cottonseed cake, although the i.v. of cottonseed oil is higher than that of groundnut oil, the reason being that the unsaturated acids of groundnut oil consist of 55 % oleic and 20–25 % of linoleic acid, whilst in those of cottonseed oil the proportion is almost reversed (about 30 % of oleic and 45–50 % of linoleic acid).

Palm-kernel oil which was fed to group V contains about 45 % of combined lauric and 15–20 % combined myristic acids, with smaller amounts of other saturated acids and a comparatively small amount of oleic acid (about 15 %); of a complex mixture of mixed glycerides, dilauromyristin and laurodimyristin are the most prominent. The chief effects on the milk fat of feeding palm-kernel oil are a slight fall in oleic, butyric and palmitic acids, a threefold increase of lauric acid and a 20 % increase of myristic acid. This suggests that milk fat of the normal type preponderates but is modified by admixture with a small amount of certain of the palm-kernel oil glycerides, mainly lauromyristins. Hilditch & Sleightholme [1930] observed a similar effect as the result of feeding cows with coconut cake. This is interesting chiefly because it shows that two fats of the same i.v. (palm-kernel oil and hydrogenated groundnut oil, i.v. 17) may lead to milk fats in which the detailed fatty acid compositions reveal quite different mechanisms of production.

The milk fats from cows of group IV, in which the hydrogenated groundnut oil of i.v. 17 supplemented the basal diet, have a fatty acid composition which more closely resembles that of the control milk fat than any of the preceding groups; the oleic acid is slightly increased (as in the work of Brown *et al.* [1941], when twice our amount of the highly hydrogenated fat was fed); the palmitic acid and the lower saturated acids are almost unchanged

(within the range of experimental error), and there is a definite increase in stearic acid. We can explain this as follows. About 50 % of the glycerides of hydrogenated groundnut oil (i.v. 17) are completely saturated (tristearin, palmito-, arachido-, etc., distearins), the remaining 50 % being mono-oleo-disaturated glycerides (oleodistearin, oleopalmitostearin, oleostearoarachidin, etc.). The fully saturated triglycerides, M.P. 65–70° and above, will not occur in the liquid phase in the cow's digestive tract unless held in solution by the mono-oleo-glycerides which are liquid at the body temperature of the cow. Arnschlick [1889] and Bloor [1914] showed that 90 % of tristearin, if fed to animals, is excreted unchanged, whilst Langworthy [1923] observed that assimilation of hydrogenated groundnut oils falls off rapidly when their melting-point exceeds 50°. With our hydrogenated groundnut oil, M.P. 58°, the bulk of the completely saturated glycerides would therefore not pass into the blood stream, and what did do so would be mono-oleo-disaturated glycerides of which oleodistearin would be a main component. Any alteration in milk fat due to this fat supplement would therefore be smaller than those associated with the softer groundnut oils fed to groups II and III. Also, since the dioleo-glycerides (and triolein) of the softer groundnut oils are absent from the hydrogenated oil (i.v. 17), which contains instead mono-oleo- (and *iso*-oleo-) glycerides (rich in oleodistearin), the slight increases in oleic acid and stearic acid (see Table 3) in the milk fats of group IV are consistent with absorption from the blood of minor quantities of glycerides such as oleodistearin.

Variation in the component acids of milk fats following ingestion of supplementary dietary fats of different degrees of unsaturation can be interpreted by taking into consideration (i) the limits of assimilation of high melting (fully saturated) components, (ii) the proportions of dioleo- and of mono-oleo-glycerides in the supplementary fat, (iii) the relatively small conversion of linoleo-glycerides and certain other acylglycerides into cow milk fats, and (iv) the experimentally justified postulate that milk glycerides are derived from blood glycerides in the mammary gland. It is unnecessary to presuppose that a desaturation of stearic to oleic glycerides is responsible for these changes.

The general trend of the present results is perhaps still more clearly discerned from the molar percentages of component acids included in Table 3. In the control fat I the molar content of palmitic acid is 23·4 % and of oleic acid 28·6 %. Groundnut oil (fed to group II) contains palmito-diolein which has 66·7 % (mol.) of oleic acid among its component acids, and in the milk fat of group II the oleic acid has increased by 6·6 % (mol.) as compared with group I though there is no significant alteration in the molar content of palmitic acid. The hydrogenated groundnut oil of i.v. 45, used in group III, consists of about 65 % of dioleo-glycerides (with 66·7 % (mol.) oleic acid) and about 25 % of mono-oleo- (and *iso*-oleo-) glycerides (with 33·3 % (mol.) oleic acid); the dioleo-glycerides include stearo- as well as palmito-diolein, and possibly some oleodistearin as well as oleopalmitostearin, owing to hydrogenation. The milk fat of group III shows, correspondingly, a smaller increase than group II in oleic acid (5·8 % mol.) as compared with the control, while the molar content of palmitic acid is decreased by 2·4 % (mol.) and that of stearic acid is increased by 4·7 % (mol.). The unsaturated glycerides in the more completely hydrogenated groundnut oil (i.v. 17) used in group IV are wholly of the mono-oleo- (or *iso*-oleo-) type and there is a correspondingly smaller increase (1·4 % mol.) in the milk-fat oleic acid as compared with the control, and a similar slight increase (1·9 % mol.) in that of stearic acid. Finally, in group V, with palm-kernel oil, the predominating lauromyristins of the dietary fat have been transferred to some extent to the milk fat, leading to a slight reduction in the oleic acid and a definite reduction (2·8 % mol.) in the palmitic acid content.

SUMMARY

1. Cows have been fed on a basal diet and on a basal diet supplemented by (*a*) groundnut oil, (*b*) groundnut oil hydrogenated to about the same i.v. as a soft butterfat, (*c*) groundnut oil hydrogenated to i.v. 17, and (*d*) palm-kernel oil, also of i.v. 17. The milk fats from the five groups have been analysed by ester-fractionation and the proportions of their component acids determined.

2. Ingestion of groundnut oil and of hydrogenated groundnut oil of i.v. 45, both of which contained considerably more oleic acid than the control milk fat, led to increased amounts of oleo-glycerides in the resultant milk fats and some diminution of the butyric-decanoic glycerides. In the case of the hydrogenated groundnut oil there was also an increase in the stearo-glycerides. Ingestion of palm-kernel oil increased the proportions of lauro- and myristo-glycerides, and slightly diminished both the palmito-glycerides and the oleo-glycerides of the resulting milk fat.

3. Ingestion of the more completely hydrogenated groundnut oil of i.v. 17 gave a milk fat almost identical with the control milk fat except for slightly increased proportions of oleic and stearic acids. This is consistent with the likelihood that this fat, about 50 % of which was completely saturated tristearin or acyldistearins melting at 65–70° or above, would be relatively unassimilable, and that those glycerides which passed into the

blood stream would be exclusively mono-oleo- (or *iso*-oleo-) glycerides, amongst which oleo- (or *iso*-oleo-) distearin would be prominent. There is no evidence in this work that any desaturation of stearo-glycerides occurs during the transformation of hydrogenated groundnut oil to glycerides in the milk of the cow.

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Sclerotiose—a Polysaccharide Metabolic Product of *Penicillium Sclerotiorum* van Beyma

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During work on the metabolism of *Penicillium sclerotiorum*, disappearance of the carbohydrate constituent of the acid Czapek-Dox and other media employed was observed [Curtin & Reilly, 1940; Reilly & Curtin, 1943]. This, it was suggested, was probably the most important factor in the metabolic process. Had the disappearance of the carbohydrate been accompanied by a rapid and permanent decrease in the *p*H, the production of an acid in the substrate would have been indicated. It was invariably found, however, in batches grown on a number of different media, that though a drop in *p*H occurred initially, the *p*H of the fully grown substrate reverted towards neutrality. That there was no connexion between these variations in *p*H and (*a*) the initial *p*H of the medium or (*b*) the production of sclerotiorine by the mould, was proved by the fact that similar series of *p*H readings were obtained when the mould was incubated (1) on acid Czapek-Dox medium (*p*H 4·2) with the production of sclerotiorine, and (2) on Raulin-Dierckx medium (*p*H 7·0) (when sclerotiorine is not produced). The filtered substrate of the mould after a full period of growth on acid Czapek-Dox medium contained no reducing carbohydrate. This, coupled with the fact that elimination of glucose caused no permanent drop in the *p*H value, suggested production by the mould of an insoluble glucose complex.

The mycelium was therefore extracted with boiling water and the flocculent precipitate which appeared on cooling was found to be a polyglucose, of specific rotation $\alpha_D = +244$ to $+284$, and for which we suggest the name '*sclerotiose*'. In properties, it proved to be similar to other polysaccharides isolated from the lower fungi, being soluble only in hot water, giving no colour with iodine, and dissolving only to a slight extent in dilute NaOH, thus resembling mycodextran from *P. expansum* [Dox & Niedig, 1914], the polysaccharides from *P. digitatum* and *Fumago vagens* [Birkinshaw, Charles & Raistrick, 1931], and the polysaccharide from *P. javanicum* [Ward, Lockwood, May & Herrick, 1935] for which the specific rotations range from $\alpha_D = +217$ to $+299$. An examination has been made of sclerotiose and of some of its derivatives.

METHODS AND DISCUSSION

Over a period of 24 days, 40 bottles, each containing 100 ml. of substrate, were inoculated and grown as described earlier [Reilly & Curtin, 1943]. At intervals of 3 days, three representative cultures were filtered, adjusted where necessary to a vol. of 300 ml., and examined by the polarimeter in a 20 cm. tube. These figures are given in Table 1.

Table 1. *Influence of growth of* P. sclerotiorum *on optical activity of acid Czapek-Dox medium*

Days of incubation	Polarimeter reading	Days of incubation	Polarimeter reading
0	+5·7°	9	+0·4°
3	+5·0°	12	+0·1°
6	+1·3°	15–24	0·0°

A batch of cultures of *P. sclerotiorum* grown on Raulin-Dierckx medium was then grown for a similar period of 24 days, and *p*H readings taken as above for three representative cultures every 3 days. The figures are given in Table 2.

Table 2. *Influence of growth of* P. sclerotiorum *on* p*H of Raulin-Dierckx medium*

Days of incubation	*p*H (colorimetric)	Days of incubation	*p*H (colorimetric)
0	7·0	15	3·1
3	5·4	18	3·6
6	4·2	21	4·7
9	2·4	24	5·1
12	2·6		

This table shows that the gradual drop in *p*H, followed by a rise towards neutrality, is not determined by the initial *p*H of the medium used.

For the isolation of sclerotiose, the bulk of the mycelium was grown for 24 days at 25° on the acid Czapek-Dox medium (glucose, 50 g.; KH_2PO_4, 1·0 g.; $NaNO_3$, 2·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water to 1000 ml.). It was also grown on certain modifications of this medium.

The mycelium was repeatedly extracted with boiling water until, on cooling, there was no further separation of flocculent material. The red aqueous extract was allowed to stand overnight, the supernatant liquid was then decanted and the precipitate separated by centrifuging. The red-tinted slimy product was washed by suspending in 2000 ml. distilled water and filtering through a double filter, on which had been deposited a uniform layer of HCl-extracted, calcined kieselguhr. It was then removed from the filter by eluting with boiling distilled water. The coloured solution was treated with norit-charcoal at 100° for ½ hr., the operation being repeated two or three times. The charcoal was finally removed by filtering through a silica plate. Sclerotiose now separated from the cooled liquid in a pure white flocculent mass. It was redissolved in fresh distilled water, allowed to settle and redissolved in fresh solvent, the procedure being repeated 10 to 15 times. The filtered product was washed with absolute ethanol, allowed to stand overnight in contact with the latter, and then washed with absolute ether and dried in a high vacuum. The pure, white, amorphous compound turned brown on heating above 260°, fused at 290–300°, and gave no residue on ignition. The yield of polysaccharide was 2·8–3·0 g./100 g. mycelium. (Found: C, 40·4; H, 6·8; O, by difference, 52·8%. $(C_6H_{10}O_5)_n.(H_2O)_n$ requires C, 40·0; H, 6·7; O, 53·3%.) The molecule of water was removed by drying *in vacuo* over P_2O_5 at 90° for 2 hr. (Found: C, 44·1; H, 6·26%. $(C_6H_{10}O_5)_n$ requires C, 44·4; H, 6·17%.)

As already stated, the bulk of the mycelium extracted was grown on the acid Czapek-Dox medium. There were, however, some samples of mycelium, grown on modifications of this substrate, in stock, and these were extracted in the same way. On neutral Czapek-Dox medium (in which K_2HPO_4 replaces KH_2PO_4) the yield of polysaccharide was 10·1% of the extracted mycelium weight, and on the same medium, but with KBr replacing KCl, the yield was 10·0%. On acid Czapek-Dox medium, from which KCl was omitted, the quantity of sclerotiose was the same as when KCl was present, i.e. 2·9% of the mycelium weight. With the Raulin-Thom medium (glucose, 50 g.; NH_4 tartrate, 4·0 g.; K_2CO_3, 0·6 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; $MgCO_3$, 0·4 g.; $ZnSO_4.7H_2O$, 0·07 g.; $FeSO_4.7H_2O$, 0·07 g.; distilled water to 1500 ml.), the mould yielded 2·4% polysaccharide. On the Raulin-Dierckx substrate (glucose, 46·6 g.; $(NH_4)_2SO_4$, 0·16 g.; K_2CO_3, 0·4 g.; $(NH_4)_2.HPO_4$, 0·4 g.; $ZnSO_4.7H_2O$, 0·04 g.; $FeSO_4.7H_2O$, 0·04 g.; 66·7 ml. of a solution of 0·71 g. tartaric acid and 0·4 g. $MgCO_3$ in 100 ml. distilled water, adjusting to 1000 ml.), the yield was negligible. The contrast between the acid and neutral Czapek-Dox media in the production of sclerotiose finds a parallel in the different rates of KCl metabolism described in the recent communication of Reilly & Curtin [1943].

Properties of sclerotiose

Sclerotiose is insoluble in all organic solvents and in cold water, but dissolves readily in hot water. It is slightly soluble in warm dilute NaOH, and in formamide solution. The solution in water gives no coloration with iodine and does not reduce Fehling's solution on boiling. It gives Molisch's carbohydrate reaction but does not precipitate lead acetate from solution. A boiling solution of sclerotiose turns aniline-acetate-paper pink. With phloroglucinol, it gives a yellow coloration in the cold, which turns brown on boiling. The products of acid hydrolysis, after neutralization with NaOH, reduce both Fehling's and Pavy's solutions. On hydrolysing with dilute HCl, adding excess NaOH and heating, an aldose resin is formed.

If the moist solid is dried without using ethanol and ether, a grey-black, horny mass is obtained which is insoluble in water, even on prolonged heating. On testing with Pavy's solution, no hydrolysis could be detected when sclerotiose was treated with dilute NaOH at 70°.

The specific rotation was found to be $[\alpha]_D = +244$ at 70° in 6% NaOH and $[\alpha]_D = +284$ at 90° in formamide.

Hydrolysis of sclerotiose

This polysaccharide was shown to be a polyglucose as follows: Sclerotiose (1 g.) was refluxed with 25 ml. *N* H_2SO_4 for 6 hr., 25 ml. *N* NaOH

were then added, together with 6 ml. phenylhydrazine and 3 ml. glacial acetic acid. The mixture was heated on the water-bath for 30 min. and the osazone which separated out in yellow needles was filtered off and recrystallized. It melted at 206° when heated quickly, and when 0·2 g. was dissolved in 4 ml. pyridine and 6 ml. ethanol, the rotation was $\alpha_D = +1{\cdot}46$. Neuburg [1899] states that for glucosazone under similar conditions $\alpha_D = +1{\cdot}50$.

Further confirmation was obtained as follows: sclerotiose (0·5 g.) was refluxed for 6 hr. with 25 ml. water and 25 ml. *N* H_2SO_4. The liquid was then titrated, with phenolphthalein as an indicator, with 24·7 ml. *N* NaOH and the volume adjusted to 100 ml. The glucose content was then estimated (1) polarimetrically, which gave 0·52 g.; (2) by the iodine method of Willstätter & Schudel [1918] as modified by Judd [1920], the result then being 0·52 g. using the Baker & Houlton [1920] value of 1 g. glucose equivalent to 1·41 g. iodine; (3) by means of Fehling's solution when the value was 0·50 g.

If we assume the first two results to be correct, the glucose produced from 0·5 g. of pure, dry, ash-free sclerotiose is 0·52 g., the calculated figure being 0·55 g.

Nitration of sclerotiose

The method was that employed by Pictet & Reilly [1921] for the nitration of levulosan. Finely powdered polysaccharide (1 part) was added gradually with vigorous stirring at room temperature, and without appreciable rise, to 50 parts of a mixture of 3 parts by weight H_2SO_4 (sp. gr. 1·84) and 1 part by weight HNO_3 (sp. gr. 1·42). The mixture was allowed to stand overnight. The compound did not dissolve during the esterification process and showed little physical change. The nitrate was boiled for a short time with slightly acid water to decompose any small amount of sulphate formed, and was then washed with distilled water and finally dried *in vacuo* over concentrated H_2SO_4 when it gave a pure white solid, which became brown at 165° and decomposed at about 200°. (Found: C, 26·8; H, 3·1; N, 11·9; O, by difference, 57·8%. $C_{24}H_{31}O_{11}(NO_3)_9$ requires C, 27·2; H, 3; N, 11·9.)

Properties of sclerotiose nitrate. This nitrate is insoluble in boiling water, dilute acid and ethanol; it is soluble in acetone, amyl and ethyl acetates, and in nitrobenzene and is sparingly soluble in diacetone alcohol. Like nitrocellulose of the same percentage composition, it is completely soluble in a mixture of ether and ethanol. It is highly inflammable and gives no residue when ignited on copper foil. On slow evaporation of an acetone solution, the gelatinous product which separates first is followed by a slightly opaque celluloid-like modification. When slowly heated to the decomposition point, the nitrate liberates oxides of nitrogen. The specific rotation in acetone solution is $[\alpha]_D = +119$.

An attempt to form a higher nitrate, by the use of fuming HNO_3, resulted in the production of sclerotiose endecanitrate. As before, 1 g. of the polyglucose was added to a mixture of 25 ml. H_2SO_4 (sp. gr. 1·84) and 15 ml. fuming HNO_3 (sp. gr. 1·50), the nitrate being isolated and purified as above. (Found: N, 13·2%. $C_{24}H_{29}O_9(NO_3)_{11}$ requires N, 13·5%.) It is similar in properties to the lower nitrate, but is sparingly soluble in a mixture of ethanol and ether. It decomposes at 132° with deflagration.

Treating a molecular mixture of the two nitrates with reducing agents, such as $(NH_4)_2S$ and H_2S, removes the nitrate groups. The regenerated sclerotiose so produced is not, however, absolutely pure, since it gives the diphenylamine test.

Sclerotiose diformate

0·9 g. of the pure, dry polysaccharide was added to 90 ml. of formic acid and 10 ml. concentrated H_2SO_4 with constant stirring. The polysaccharide quickly dissolved to give a slightly opaque brown solution without an appreciable rise in temperature. After standing overnight, the formate was precipitated by pouring into ice-water, was recrystallized from formic acid, and then dried *in vacuo*. This compound is soluble in pyridine and formic acid but insoluble in other organic solvents. It is also slightly soluble in thiocyanate solutions. The formic acid content was determined by slowly treating 0·5 g. of the dry formate with 75 ml. *N* $K_2Cr_2O_7$ and adding 20 ml. concentrated H_2SO_4, the mixture being kept cool. Further cooling discourages the evolution of CO_2. The mixture was then left for 8 hr. on a boiling water-bath and the excess of $K_2Cr_2O_7$ titrated with *N* $FeSO_4$ solution. (Found: 39·7% formic acid. $(H.COO)_2C_6H_8O_3$ requires 42·2%.)

Preparation of acetyl derivatives of sclerotiose

The method used was that of Reilly, Hayes & Drumm [1931]. 2 g. finely powdered polysaccharide were added to 20 g. acetic anhydride and 2 drops of concentrated H_2SO_4. Most of the solid dissolved to give a brown solution with liberation of heat. The mixture was then heated nearly to the boiling-point when complete solution was effected. The crude acetate, precipitated on pouring the reaction mixture into ice-water, was washed free from acetic acid, dried and extracted with acetone. The insoluble fraction was dissolved in chloroform. Both fractions were then shaken with norit charcoal and allowed to stand for 24 hr.

Acetone-soluble acetate. The decolorized solution, filtered free from charcoal, was poured into ice-water, when the acetate separated as a fine white powder, which did not settle out on standing. It was gravity-filtered through a silica plate. When dry it was slightly coloured. Accordingly, it was redissolved in acetone, treated with charcoal, precipitated and dried again. The pure white acetate so obtained was insoluble in water, ethyl and amyl alcohols, carbon tetrachloride and benzene, but was soluble in acetone, pyridine and glacial acetic acid. On analysis, it was found to contain 48·2% acetic acid; $C_6H_8O_3(CH_3{:}COO)_2$ requires 48·8%.

Chloroform-soluble acetate. The acetate was precipitated by pouring the faintly brown solution into ice-cold ether and the grey precipitate redissolved in $CHCl_3$ and then treated as above. On evaporating under reduced pressure microcrystals were obtained. These were further purified by dissolving in warm glacial acetic acid and precipitating with ice-water. The pure white crystalline acetate so obtained was soluble in chloroform, pyridine and glacial acetic acid but insoluble in water, acetone, ethanol, benzene and ether. It fused at 166–172° to a gelatinous mass. The compound was found to contain 61·7% acetic acid; the compound $C_6H_7O_2(CH_3.COO)_3$ requires 62·5%.

It was thought that the brown colour of the reaction mixture previously mentioned might indicate contamina-

tion by degradation products. The preparation was therefore repeated, using Barnett's method [1921] in which sulphuryl chloride is employed as a catalyst (thereby reducing degradation to a minimum) and was carried out according to the modification used by Irvine & Hirst [1922] for the preparation of cellulose tri-acetate. 2 g. sclerotiose were soaked in 10 ml. glacial acetic acid, through which a stream of dry sulphuryl chloride had been passed for 30 sec. After setting aside for ½ hr., 122 ml. of acetic anhydride were added and SO_2 was then passed through the liquid for 1 min. At the end of 1 hr., during which the pasty mixture was vigorously stirred, the temperature was raised to 65° and maintained there until the polysaccharide had all dissolved to give a clear, colourless, viscous solution. After cooling to 30°, an equal volume of $CHCl_3$ was added, followed by a large excess of water. The mixture was continuously stirred and the $CHCl_3$ evaporated. During this process the acetate separated in the form of fine white granular particles, which were obtained as an aggregate of microcrystals on slow evaporation of the $CHCl_3$ solution. M.P. 165–168° (decomp.). (Found: C, 49·6; H, 5·8; O, by difference, 44·6%. The tri-acetate $C_6H_7O_5(OC.CH_3)_3$ requires C, 50·0; H, 5·5; O, 44·5%.)

There was no appreciable loss of material by this method. The filtrate had no action on Fehling's solution, and on evaporation to dryness gave a negligible residue; it was optically inactive. The specific rotation of sclerotiose tri-acetate, in $CHCl_3$ solution, was $[\alpha]_D = +205{\cdot}7$, while the acetic acid content was 62·1% (calc. 62·5%).

Sclerotiose benzoate

Benzoylation was carried out by a modification of the Schotten-Baumann reaction. The final product had a benzoic acid equivalent of 65·6%. It was therefore probably the tri-benzoate, but contained some di-benzoate.

SUMMARY

1. Investigation of the rapid glucose consumption of *P. sclerotiorum*, on Czapek-Dox and Raulin-Thom media, led to the isolation of a polyglucose, somewhat similar to other polysaccharide metabolic products of the lower fungi.

2. The influence of the culture medium and its *p*H were amply demonstrated by the varying yield of sclerotiose. As expected the neutral medium gave the greater proportion of polyglucose, the more acid substrates having a hydrolysing effect [Reilly & Curtin, 1943]. Of the derivatives prepared, the primary or tri-derivative was always present in preponderating amount.

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The Excretion of a Metabolic Product of Salicylic Acid

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The experiments described below are a continuation of the work by Lutwak-Mann [1942].

METHODS

Alkali test in urine. It was shown previously that shortly after the injection of salicylate there appears in rat urine a substance, which on addition of fairly strong NaOH, and in the presence of air, causes the urine to turn brown to black. The following modifications of this test have now been worked out. When only very small amounts of urine are available 1–2 drops of it are added to about 4–6 ml. NaOH (*M*/50 or stronger). After a 30–60 sec. lag a pink colour develops which persists for varying lengths of time (2–5 min.), after which the solution turns yellow to brown. Excess of either urine or alkali prevents the appearance of the pink phase and only the final brown colour is seen. A still more reliable result is obtained by acidifying a few ml. of urine with 10% HCl and shaking it with 3 vol. ether. The separated ether extract is then shaken with a few ml. dilute NaOH. The aqueous solution turns at once a bright pink colour which persists for a short while and then the

solution becomes brown. A very small trace of the alkali-sensitive substance excreted in urine after the administration of salicylates can be detected in this manner.

Isolation from urine of the substance responsible for the alkali test. Six large male rats (300 g.) were injected every second day with a mixture of 0·3 mg. Na-salicylate plus 0·3 mg. Na-aspirin/g. wt. until the animals had received a total of 3·2 g. salicylates. The urine collected during this period (450 ml.) was acidified with HCl and extracted with ether repeatedly till a sample of the extracted urine failed to give a positive alkali test. The ether extract was washed with dilute $NaHCO_3$ till the washings became colourless. The aqueous solution was divided into two, half of it was acidified and again extracted with ether (fraction A). The rest was adjusted to *p*H 6·8 and Pb acetate was added, avoiding excess. The Pb precipitate was discarded as it was found that the supernatant alone gave the alkali test. The Pb-containing fluid was left at room temperature for 2 days when it was found that the alkali-sensitive substance could no longer be extracted by ether though it could be extracted with ethyl acetate (fraction B).

Fraction A contained a great deal of salicylic acid as well as the substance responsible for the NaOH test. It was noticed, however, at this stage, that when dilute $FeCl_3$ was added to a sample a distinct blue tinge developed though it was somewhat obscured by the purple colour characteristic of salicylic acid. When salicylic acid was removed by extraction with chloroform and benzene, a whitish powder (0·15 g.) was obtained from this fraction, which showed the following properties. It was acid in aqueous solution, and turned intensely blue with dilute $FeCl_3$; on addition of NaOH it became pink and then slowly went brown, thus resembling the reactive substance in the urine. It reduced Fehling's solution on heating, ammoniacal $AgNO_3$ in the cold, and phosphomolybdic acid on prolonged standing. It was fairly soluble in hot water, very soluble in ethanol and ether, but insoluble in chloroform, benzene and CS_2. On recrystallization from water the M.P. 198° was obtained. The properties of the substance isolated which is responsible for the behaviour of salicylate urine towards NaOH, resemble those of 2:5-dihydroxybenzoic or gentisic acid. Using a specimen of gentisic acid prepared by the method of Graebe & Martz [1905] which showed a M.P. 199°, a mixed M.P. of 198° was obtained; owing to the author's having taken up war work it was not possible to carry out the full analysis of the product obtained from urine. The assumption, however, that this substance is gentisic acid is further strengthened both by the older findings of Angelico [1921], Baldoni [1908], and Neuberg [1911], as well as by the recent work of Kapp & Coburn [1942], who describe the isolation and identification of gentisic acid from human urine as one of the urinary metabolites of Na-salicylate.

From the Pb-containing ethyl-acetate fraction B, after washing with water, a solution was obtained which gave a strong test with NaOH and reduced Fehling's reagent, but which gave only a pinkish brown colour with $FeCl_3$. When this fraction was concentrated on a water-bath and cooled in ice it turned into a transparent gel, which subsequently solidified into a white crystalline mass. After recrystallization from dilute ethanol 0·48 g. of white crystalline needles was obtained. The behaviour of this substance towards NaOH and Fehling's reagent, as well as its extremely poor solubility in cold water, are analogous to those of the Pb salt of gentisic acid [Senhofer & Sarlay, 1882]. For reasons mentioned above, however, its analysis could not be completed.

An attempt was made to show the *in vitro* formation of gentisic acid from salicylate. The following samples were set up and incubated for 2 hr. at 38° in $O_2 + CO_2$ (95 + 5%): (*a*) 25 g. liver slices (rat), (*b*) 18 g. liver pulp, (*c*) 15 g. minced skeletal muscle, all of them containing *M*/50 salicylate, *M*/30 phosphate buffer of *p*H 7·3, and 0·3% bicarbonate. From the acid-ether extracts of the muscle mince and the liver pulp a chloroform-insoluble fraction was obtained which, dissolved in ethanol, gave a distinctly blue colour with dilute $FeCl_3$, and slightly darkened on addition of NaOH. No such definite result could be obtained in the extracts from the liver slices where substances may have been present which interfered with these tests.

The substance isolated from urine, presumably gentisic acid, was examined and found to be stable in the presence of the enzymes of rat liver, kidney and muscle, as well as of baker's yeast. It was not oxidized by cytochrome or catechol oxidase. Peroxidase slowly but steadily oxidized it in presence of H_2O_2.

Substances which give the alkali test in urine. The following substances related to salicylic acid were administered to rats: acetylsalicylate (Na-aspirin), methylsalicylate (oil of wintergreen), phenylsalicylate (salol), Na-sulphosalicylate. Their effect on the urine excretion was examined as described in previous work. All of them caused an increase in the reducing power of urine (as measured by titration against 2:6-dichlorophenol indophenol) presumably owing to an increased excretion of ascorbic acid, but the NaOH test was positive only after aspirin and methylsalicylate injections. The alkali test in urine after aspirin injection was always stronger than after a similar dose of salicylate. After the injection of methylsalicylate early urine samples (0–7 hr.) did not exhibit a positive alkali test as they did after salicylate or aspirin treatment; the later samples, however (7–24 hr.), showed a strongly positive test.

Substances which prevent the formation of gentisic acid from salicylates. Previous work has shown that large doses of ascorbic acid are without effect on the excretion in urine of the substance formed from salicylic acid which is responsible for the alkali test, though such doses completely abolished the excretion of homogentisic acid in tyrosine alkaptonuria. In this work the effect was studied of poisons such as white phosphorus (P), carbon tetrachloride (CCl_4) and phlorrhizin upon the excretion of gentisic acid after salicylate treatment.

(*a*) *White phosphorus.* A group of 10 rats received 0·1 mg. white P in olive oil every 2 days for a fortnight. At the end of this period autopsy showed fatty infiltration of the liver; the titratable reducing power of the urine increased considerably, and all the rats excreted large amounts of urobilin. Some of them were then injected with salicylate, others with Na-aspirin (0·5 mg./g.). Urine analysis showed the following facts: that no NaOH test was

given, either soon after the injection or in later urine samples; that the titratable reducing power was still further increased, but that there was no rise in the *p*H of the early samples and no albuminuria, though these were both previously found to be characteristic features of urine from salicylate-treated normal rats.

The effect of acute P poisoning was also studied. The rats received only 2 doses of P (1·5 mg.) in the course of 3 days, after which they were given salicylate and aspirin by injection. Although there was no urobilinuria, and as yet no marked changes in the liver on autopsy, these rats failed entirely to excrete gentisic acid, as judged by negative alkali tests in their urine.

Both the chronically and the acutely poisoned rats were kept for another 10 days on a normal diet without white P. Urobilinuria continued unabated, but the majority of the animals survived, and those rats which had been treated with salicylates seemed to recuperate more quickly than the others. At the end of this period the injections of salicylate and aspirin were repeated. This time all of them excreted urine which gave a strongly positive test with NaOH, i.e. recovery had occurred.

(*b*) *Carbon tetrachloride.* Injections of CCl_4 (0·4 ml./200 g.) were given every other day to rats. After 14 days of this treatment, which was remarkably well tolerated, they received injections of salicylate and aspirin (0·5 mg./g.). Of eleven CCl_4-poisoned rats, eight failed to excrete the substance responsible for the alkali test, but the urine of the remaining three animals showed a faintly positive test with NaOH. The titratable reducing power of the urine, which fell conspicuously during the CCl_4 treatment, rose in the usual manner in response to salicylates. When the CCl_4 injections were discontinued for a few days and the administration of salicylate and aspirin repeated, all eleven rats excreted urine which gave a strong NaOH test.

Experiments were made with the P- and CCl_4-poisoned rats in which anthranilate (*o*-amino-benzoate) was used instead of salicylate. It had been shown previously that the administration of anthranilate to rats caused the excretion in urine of a substance which in presence of alkali (NH_4OH) turned purple, and that in addition the urine contained considerable quantities of a glucuronide. When the poisoned rats were given anthranilate they continued to produce the glucuronide, but the test with NH_4OH was completely negative.

(*c*) *Phlorrhizin.* This was given by injection (30 mg./200 g.). Three hours later when glucosuria was fully developed some of the rats were given salicylate by injection, others aspirin (0·5 mg./g.). The NaOH test was greatly increased in all urines, and that from the aspirin-treated rats turned almost black on addition of NaOH. The titratable reducing power, however, was rather lower than in non-phlorrhizinized animals.

The alkali test in human urine. Urine was collected and examined after an oral dose of aspirin (1·2 g.). The acid-ether extract from the early urine samples (0–7 hr.) showed no alkali test, but the 7–15 hr. samples, which also showed a slight increase in the titratable reducing power, gave a definite test. Later samples (15–24 hr.) showed only a mere trace of the test.

SUMMARY

1. A substance previously reported to be present in rat urine after the administration of salicylate, and which causes the urine to turn dark on addition of NaOH in air, has been isolated. Its properties are found to agree with those of 2:5-dihydroxybenzoic acid (gentisic acid).

2. Tests are described by means of which traces of this substance can be demonstrated in urine.

3. Positive tests of this kind were obtained after the administration of salicylate, aspirin and methylsalicylate, but not after salol or sulphosalicylate. Human urine gave a positive test after a comparatively small dose of aspirin.

4. The substance isolated from urine of salicylate-treated rats, which is thought to be gentisic acid, is stable in the presence of rat tissue, and of yeast enzymes. It was oxidized by peroxidase, but not by cytochrome or by catechol oxidase.

5. Rats poisoned with white phosphorus or carbon tetrachloride are unable to excrete gentisic acid after administration of salicylates. However, the effects of the poisoning are not permanent, since when the poisons are discontinued for a period, recovery sets in and administration of salicylates is then again followed by the excretion of this particular product of salicylate metabolism. On the other hand, phlorrhizin does not prevent, but rather increases, the excretion of the alkali-sensitive substance in the urine after administration of salicylates.

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On the Failure of Azo-gelatin as an Antigen

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The non-antigenicity of gelatin was attributed for a long time to its lack of aromatic amino-acids. Later, Landsteiner [1919] and Hooker & Boyd [1932] introduced aromatic determinant groups into the gelatin molecule by coupling it with diazotized arsanilic acid. Injection of arsanil-azo-gelatin into rabbits furnished immune sera which gave slight precipitates with the arsanil-azo-compounds of casein, ovalbumin and serum-globulin, but gave no visible reaction with arsanil-azo-gelatin. Similar results were obtained by coupling gelatin with glucosido-tyrosine [Clutton, Harington & Yuill, 1938]. These experiments demonstrated clearly that the absence of antigenicity in gelatin cannot be attributed merely to the deficiency of aromatic groups.

In previous papers [Haurowitz, 1936; Haurowitz & Kraus, 1936] we showed that the bulk of intravenously injected arsanil-azo-globulin is fixed in the liver and the bone-marrow of rabbits. We have now performed similar experiments with arsanil-azo-gelatin.

METHODS

Arsanil-azo-globulin was prepared in the usual way by coupling pseudo-globulin from sheep serum with diazotized arsanilic acid [Haurowitz, 1936]. For arsanil-azo-gelatin 1·35 g. of arsanilic acid were diazotized in the usual manner [Haurowitz, 1936], and the solution added to a chilled solution of 4·5 g. of gelatin in 140 ml. of 0·65 *N* NaOH. The mixture was permitted to stand at 0° for 16 hr. and then neutralized, precipitated by twice its volume of acetone, dissolved in water and dialysed for 48 hr. against distilled water. The solutions for injection into rabbits contained 2–3 % of azo-protein. The intravenous injection into rabbits and the determination of arsenic in the organs have been described in previous papers [Haurowitz, 1936; Haurowitz & Kraus, 1936; Haurowitz & Breinl, 1932]. The animals were killed by bleeding 60 min. after the injection of the azo-protein.

RESULTS

Deposition of arsenic in the liver. As will be noted from Table 1 only 4·4 % of the injected arsenic was deposited in the liver, whereas 66 % passed into the urine. In a second rabbit, given an injection of arsanil-azo-globulin under similar conditions, 34 % of the injected arsenic was found in the liver and only 9 % passed into the urine. A repetition of this experiment on two other animals furnished analogous results.

Excretion of arsenic. The experiments recorded in Table 2 were performed on rabbits weighing 1·7–2·2 kg. In each experiment a pair of animals was used, since the food varied somewhat from day to day, both animals of a pair receiving the same food. One animal received an intravenous injection of the arsanil-azo-gelatin, the other animal received arsanil-azo-globulin as a control. The animals were kept in metabolism cages and the urines collected quantitatively during 2 days. Arsenic was determined in 10 or in 20 ml. of urine according to our previously described method.

Table 1. *Distribution of arsenic 60 min. after the intravenous injection of arsanil-azo-gelatin and arsanil-azo-globulin into rabbits*

Injected protein	50 mg. of arsanil-azo-gelatin (=1·8 mg. As)	60 mg. of arsanil-azo-sheep serum globulin (=0·96 mg. As)
Wt. of rabbit	2200 g.	1760 g.
Liver: (*a*) Weight	67 g.	57·5 g.
(*b*) Content of As	0·08 mg. (=4·4 % of injected As)	0·33 mg. (=34 % of injected As)
Bone marrow	0·25 mg. As/100 g.	0·71 mg. As/100 g.
Blood	0·025 mg. As/100 ml.	0·30 mg. As/100 ml.
Kidneys	0·075 mg. As	0·072 mg. As
Urine: (*a*) Volume	9·5 ml.	7·0 ml.
(*b*) Content of As	1·2 mg. (=66 % of injected As)	0·088 mg. (=9·2 % of injected As)

In each of these experiments arsenic was eliminated more rapidly by the animals which had been injected with arsanil-azo-gelatin (Table 2).

The rapid elimination of arsenic after the injection of arsanil-azo-gelatin cannot be attributed to the presence of dialysable compounds of arsenic in the injected preparation, for it had been purified by dialysis, and further dialysis in distilled water

Table 2. *Excretion of arsenic after the injection of arsanil-azo-gelatin and arsanil-azo-globulin*

Exp. no.	Rabbit no.	Injected arsanil-azo-protein: Wt. mg.	Protein	Content of As mg.	Content of arsenic in urine: 24 hr. mg.	48 hr. mg.
1	208	60	Globulin	2·5	0·91	—
	209*a*	50	Gelatin	1·8	1·54	—
2	210	60	Globulin	2·5	0·88	—
	209*b*	50	Gelatin	1·8	1·71	—
3	213	48	Globulin	2·0	0·28	0·06
	214	45	Gelatin	1·6	0·99	0·05
4	215	48	Globulin	2·0	0·47	0·02
	217	56	Gelatin	2·0	0·75	0·07

yielded only negligible quantities of dialysable As compounds.

The rapid elimination of As when administered intravenously as arsanil-azo-gelatin appears to be related to the peculiarities of the gelatin molecule, e.g. its strong tendency to remain in solution and not to be adsorbed. It is assumed therefore, that the failure of arsanil-azo-gelatin to produce antibodies is chiefly due to the fact that its molecules are not deposited at the sites of antibody-formation, but are rapidly eliminated from the organism. Probably this is also the reason for the lack of antigenicity of pure gelatin.

SUMMARY

1. While an intravenous injection of arsanil-azo-globulin into rabbits gives rise to a considerable deposition of arsenic in the liver, an analogous injection of arsanil-azo-gelatin is followed by rapid urinary excretion of arsenic, and only small quantities of arsenic are deposited in the liver.

2. The failure of arsenil-azo-gelatin to act as full antigen is attributed to the insufficient deposition of this substance in the reticulo-endothelial cells, i.e. in the cells producing the antibodies.

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Riboflavin and Riboflavin Adenine Dinucleotide in Ox Ocular Tissues

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Sherman & Sandel [1931] found that vitamin 'G'-deficient rats showed eye changes which they described as 'a very slight keratitis with corneal corpuscles, a few polymorphs and some new formed blood channels in the cornea'. Day, Langston & O'Brien [1931] also observed that rats on a 'G'-deficient diet developed eye lesions. Their animals not only developed a condition which they described as anterior interstitial keratitis, but also cataract. The lens changes in vitamin 'G' deficiency were further investigated by many different workers, but the original observations could not be repeated. The changes in the cornea were not examined in detail until Bessey & Wolbach [1939] and El Sadr [1939] published descriptions of the vascularization of rat corneas in riboflavin deficiency. In the meantime vitamin 'G' had become identified with vitamin B_2 or riboflavin. Sydenstricker, Sebrell, Cleckley & Kruse [1940] described vascularization of the cornea in man in riboflavin deficiency. Patek, Post & Victor [1941] have found that it occurs in pigs, and Street, Cowgill & Zimmerman [1941] have described its occurrence in dogs.

Apart from estimations of the total flavin content of the retinae of different species by v. Euler & Adler [1933; 1934*a*] no determinations of the amount or type of flavin in the different parts of the eye have been published. We have now estimated total flavin and riboflavin adenine dinucleotide in ox eyes in order to see whether a determination of the distribution and type of flavin present would help to explain the sensitivity of the cornea to riboflavin deficiency.

METHODS

(1) *Collection of material*

Ox eyes were removed and put into a refrigerator within 5 min. of the death of the animal. As soon

as the requisite number of eyes had been collected they were taken on ice to the laboratory, dissected, and extracts made of the different parts. The eyes were gently wiped with a damp pledget of cotton-wool. The corneal epithelium was then shaved off with a sharp knife. We found that the best method of removing the retina was to brush it out with a soft paint brush, the choroid could then be taken out with forceps. The aqueous humour, when needed, was removed at the slaughter house with a hypodermic syringe, and immediately cooled on ice. The lens was extracted without its capsule. Ocular conjunctiva was cut off as a very thin strip without adherent fatty tissue, in order to make conjunctiva as comparable with corneal epithelium as possible.

Preparation of extracts. Snell & Strong [1939] recommend extraction of tissues low in riboflavin either with boiling $N/10$ HCl or with a mixture of 3 parts acetone, 1 part N H_2SO_4 under reflux. We found, however, that these methods gave lower values than extraction with H_2O at 80° for 15 min., which is the method used by Ochoa & Rossiter [1939] to prepare extracts for riboflavin adenine dinucleotide estimation. The final method of preparation of tissue extracts for both the microbiological estimation of total riboflavin and the estimation of riboflavin adenine dinucleotide has therefore been as follows. The tissue was weighed, ground with a little sand and extracted with distilled water in a water-bath at 80° for 15 min. The final concentration of tissue was 10 or 5%. The tube was then cooled to 4° and the extract centrifuged. The supernatant, which is frequently cloudy, was used for the estimations.

(2) *Microbiological estimation of total riboflavin*

Total riboflavin was estimated by the microbiological method of Snell & Strong [1939]. A culture of *Lactobacillus helveticus* (kindly supplied by Dr J. G. Davis of the National Institute for Research in Dairying) was used. A few experiments were done with additions to the medium as described by Barton-Wright [1942], i.e. xylose, asparagine, nicotinic acid and pantothenic acid. Although these additions did accelerate the growth of *L. helveticus* to a small extent, they did not affect the values for riboflavin obtained from different extracts of the same tissues. For the rest of the work the simplest medium was used. The growth of the *L. helveticus* in 24–48 hr. was measured by titrating the acid formed with $0{\cdot}1N$ NaOH, using phenol-red as an indicator. A standard riboflavin curve was obtained with each experiment between the limits of 0–0·1 μg. riboflavin and the extracts were diluted, if necessary, to fall within these levels. All extracts, with the exception of tears and meibomian secretion, were estimated in duplicate at three different levels.

(3) *Estimation of riboflavin adenine dinucleotide*

This was estimated by the *d*-amino oxidase technique of Warburg & Christian [1938], using the specific enzyme-protein from sheep's kidney. The pure substance was not available as a standard, so an extract of rat brain was used. Hot aqueous extracts of rat brain consistently contain a mean of $10 \pm 0{\cdot}8$ μg./g. fresh tissue, based on groups of six animals [Ochoa & Rossiter, 1939], and we have assumed a value of 10 μg./g. in constructing a reference curve for the O_2 uptake of 5 mg. of the kidney protein in the presence of *dl*-alanine and varying amounts of rat-brain extract. In assays on extracts of eye tissues the amounts added were always such as to give O_2 uptakes falling on that part of the reference curve for which the increase in uptake was directly proportional to the amount of catalyst added. It is realized that the values we have obtained are approximate only, as the brain extract cannot be as satisfactory a standard as the pure riboflavin adenine dinucleotide.

RESULTS

Table 1 summarizes the results we have obtained by the two methods.

We do not wish to stress the apparent agreement between the riboflavin values obtained by the two separate methods of estimation, as estimations of the flavin contents of other tissues made in the same way did not agree so satisfactorily. The riboflavin values obtained by the *d*-amino oxidase technique, given in Table 1, demonstrate the presence throughout the eye of riboflavin adenine dinucleotide.

Adler & v. Euler [1938] found that free riboflavin was present in an extract, prepared at 0°, of cod's eyes, but the question of the presence of free riboflavin in mammalian retinae is still open. Many fish retinae contain much more total flavin than any known mammalian retina. v. Euler & Adler [1934*a*] found 0·2–0·4 μg. riboflavin/g. wet wt. of human retina. We were able to obtain one normal human retina which was iced within 1 min. of extraction of the eye. The total riboflavin content, estimated microbiologically, was 3·2–5·0 μg./g. wet wt., and estimated by the *d*-amino oxidase technique was 5·0 μg./g. wet wt. Even this higher value is far below the flavin content of some fish retinae, which Adler & v. Euler [1938] found to contain 500 μg./g. wet wt.

v. Euler & Adler [1933] based their contention that the riboflavin in the retina is free on the fact that it is largely dialysable. But Pulver [1940] has shown that the flavin from all tissues becomes progressively dialysable as the time of dialysis increases, and that in order to get a true value for free riboflavin an ultrafiltrate of a press juice of the

Table 1. *Riboflavin in ocular tissues estimated by microbiological and* d-*amino oxidase techniques*

Tissue	Extractive	Riboflavin μg./g. wet wt. unless stated otherwise: Microbiological	*d*-amino oxidase (H_2O extracts only)
Corneal epithelium	*N*/10 HCl	1·76	1·8, 1·9, 2·5, 2·4, 2·3
	N/10 HCl	2·4	2·7
	Acetone, H_2SO_4	1·6	
	H_2O	1·5	1·7
Substantia propria	*N*/10 HCl	0·27	
	Acetone, H_2SO_4	0·18	0·42, 0·26, 0·25
	H_2O	0·24	0·54
Conjunctiva	*N*/10 HCl	0·9	2·6
	H_2O	1·6	
	H_2O	1·4	2·1
	H_2O	2·1	2·1
	H_2O	2·6	2·1
Aqueous humour	*N*/10 HCl	1·0 μg./100 ml.	1·3 μg./100 ml.
	N/10 HCl	0·9 μg./100 ml.	
	N/10 HCl	1·1 μg./100 ml.	
Iris	H_2O	1·4	1·2, 2·2
	H_2O	1·8	2·8
Ciliary body	H_2O	2·0	1·8
	H_2O	1·8	2·6
			1·6, 2·9
Lens	H_2O	0·05	0·28
	H_2O	0·12	0·2
Vitreous humour	Acetone	0·8 μg./100 ml.	
Retina	*N*/10 HCl	0·9	2·8, 2·1, 4·4
	N/10 HCl	1·1	3·9, 3·9
	N/10 HCl	2·1	
	H_2O	4·6	3·6, 3·8
	H_2O	4·3	1·5
	H_2O	4·9	
	H_2O	3·5	4·5
Choroid	*N*/10 HCl	0·9	1·8, 2·5, 2·8
	N/10 HCl	0·9	
	N/10 HCl	1·1	
	H_2O	1·8	2·8
	H_2O	2·9	1·5
		3·2	1·4

In those cases where a dotted line is drawn between the figures, the microbiological and the *d*-amino oxidase estimations were carried out on the same extract.

tissue should be made. Using this method he found, for example, that only 2% of the total flavin of cattle liver was dialysable.

v. Euler & Adler [1934*a*] considered that the retinal flavin was almost entirely confined to the pigment epithelium of the retina. In our experiments histological preparations showed that the pigment epithelium remained attached to the choroid, and none of it was present in the retinal preparations. Yet the flavin content of the ox retina is higher than that of the choroid. Acetone extracts of calf retina made by v. Euler & Adler [1934*b*] gave non-fluorescent extracts, which after standing for 2 days fluoresced blue. This suggests a breakdown of a combined flavin present in the original extract.

We have done a single experiment to see whether bleaching of the retina caused any diminution of its riboflavin adenine dinucleotide content. An excised ox eye was placed in a dish of ice and exposed to direct sunlight for 1 hr. At the end of this time the dinucleotide content of the retina, determined in the usual way, was normal in amount.

In Table 1, the low value for the flavin of the aqueous humour is very noticeable; that for the *substantia propria* of the cornea is also low, while the ocular conjunctiva and the corneal epithelium contain about 2 μg./g. of total flavin. Histological preparations showed that very little *substantia propria* was detached with the corneal epithelium. This accumulation of flavin in the corneal epithelium, which is without a blood supply, led us to investigate the flavin content of the glands of the eye and their secretions.

Table 2. *Riboflavin in ocular glands and secretions, estimated by microbiological and* d-*amino oxidase techniques. H_2O used as extractive throughout*

Tissue	Riboflavin, μg./g. wet wt. unless stated otherwise: Microbiological	*d*-amino oxidase	
Meibomian glands	3·9	4·1	
		4·3	
Lacrymal glands	6·5	6·3	
		6·2, 7·2	
Meibomian secretion	4·1, 3·3		
	0·0, 3·1		
Tears: Human	0·01 μg.	0·027 μg.	Total riboflavin. Vol. of tears, approx. 0·2 ml. in each case
Rabbit	0·03 μg.		
Rat	0·04 μg.		

There are two main lacrymal glands in the ox; both had similar flavin contents, averaging 6·5 μg./g. The meibomian glands of the lids contained about 4 μg./g. These values, in particular that for the lacrymal glands, are higher than those for the tissues adjacent to the corneal epithelium, or indeed for any part of the eye except possibly the retina. We therefore estimated the total flavin content of the eye secretions. Ox eyelids were cut off immediately after death and the secretion from the meibomian glands was expressed by hand. A few mg. of a hard bright yellow waxy substance was obtained from several lids. Too little was obtained to make it feasible to do parallel estimations of total flavin and riboflavin adenine dinucleotide, so that values for total flavin only are given (Table 2). These show that the secretion contains about the same amount of flavin/g. as the gland itself. Miscellaneous estimations of the presence of flavin in the tears of different species have been carried out. The volume of tears obtained was so small that it was only possible to decide whether flavin was present or not. The results show that flavin was present in tears from a normal woman, from rabbits, and from a rat.

It is therefore possible that the flavin of the corneal epithelium is obtained from the lacrymal

secretions rather than from the blood in the limbal loops. The weight of epithelium of an ox eye is approximately 0·15 g., which means that the amount of riboflavin present is 0·3 μg. It seems reasonable to suppose that this amount could be kept replenished by the eye secretions, but we have no evidence that *in vivo* such an absorption of either riboflavin or riboflavin adenine dinucleotide into the cornea does occur. The penetration of the corneal epithelium by either of these substances has not yet been investigated. Histological studies of the eye glands of riboflavin-deficient animals have not been reported, although both deficient and excessive secretion of tears have been noted, so that one cannot tell whether the primary lesion leading to a disturbance of the corneal epithelium with subsequent vascularization of the cornea is in the eye glands and their secretions or not. Deficiency of tears in man may rarely be associated with corneal disturbance, as in *keratoconjunctivitis sicca* described by Bruce [1941], but in general either the meibomian glands or the lacrymal gland may be removed without subsequent damage to the cornea. Under such circumstances the mucous-secreting glands of the conjunctiva still remain.

Johnson & Eckhardt [1940] reported that exposure of rats to sunlight hastened the vascularization of the cornea in riboflavin deficiency. We have investigated the stability to light of riboflavin adenine dinucleotide in ox corneal epithelium. Ox eyes were cooled to 4° and placed in a dish of ice in bright sunlight out of doors for 1 hr. The surface of the cornea was kept moist by occasional drops of ice-cold saline. The riboflavin adenine dinucleotide of the epithelium, estimated in the usual way, showed no diminution during this time. Ox eyes were also similarly exposed for a maximum time of 1 hr. to a G.E.C. Osira ultra-violet lamp placed 8 in. away from the corneal surface. Again no change occurred in the dinucleotide content of the epithelium. Excised epithelium exposed to the G.E.C. Osira lamp also had a normal dinucleotide content after exposure. These results with excised eyes give the impression that the dinucleotide itself in the ox corneal epithelium is relatively stable, but they must not be regarded as contradicting Johnson & Eckhardt's results, where continuous exposure to sunlight *in vivo* resulted in an apparent diminution of the corneal flavin.

DISCUSSION

These estimations of riboflavin and riboflavin adenine dinucleotide in the secretions and different parts of the eye show that the distribution is general, but uneven. The vitreous humour, the aqueous humour and the lens contain very small amounts of flavin. This is in marked contrast to the distribution of ascorbic acid, whose concentration in ox aqueous humour is 20 mg./100 ml. [Müller, 1933], a concentration greater than that in the blood. Birch & Dann [1933] found that ox lens, and Monajukowa & Fradkin [1935] found that the vitreous humour also had a high content of ascorbic acid. The estimations also show that some at least of the retinal and choroidal flavin in the ox is present as dinucleotide.

In riboflavin deficiency in man and animals the cornea is invaded by capillaries at an early stage. Our estimations of the flavin content of the epithelium and the surrounding tissues show that the corneal epithelium has a greater concentration of flavin than the *substantia propria* or aqueous humour, and about the same concentration as the conjunctiva. The source of this flavin in the corneal epithelium has been considered to be the blood in the vessels at the limbus. Our estimations of the flavian content of the eye glands and their secretions show that if the epithelium is permeable to flavin it would be possible for it to obtain it from tears and from the meibomian secretion, and we think that this possibility should be borne in mind in considering the ocular signs of riboflavin deficiency.

SUMMARY

Total riboflavin and riboflavin adenine dinucleotide have been estimated in ox ocular tissues. The lacrymal and meibomian glands contain a greater amount of flavin than does any other part of the eye. The corneal epithelium contains much more than the *substantia propria* or the aqueous humour, and it is tentatively suggested that the corneal epithelium may get flavin from the eye secretions as well as from the blood in the limbal capillaries.

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The Oxalate Content of Blood

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The quantity of oxalate present in blood is still in dispute. Japanese workers give the normal value as 2–4 mg./100 ml. (method of Izumi [1933], Suzuki [1934], Kamiya [1937] and Sato [1941]). On the other hand, Leulier & Dorche [1939] found 0·2–0·5 mg./100 ml. in good agreement with Flaschenträger & Müller [1938]. Voinar & Babkin [1940] failed to isolate oxalic acid from the blood or tissues (as CaC_2O_4), and expressed doubts as to the reliability of the methods then available. Barber & Gallimore [1940] applied the ester method of Dodds & Gallimore [1932] and reported 0·4–0·6 mg./100 ml. of human blood.

The method of Izumi [1933], which depends on the precipitation of cerous oxalate ($Ce_2(C_2O_4)_3$) at *p*H 2·3 from a trichloroacetic acid filtrate, received some support from the previous work of Merz & Maugeri [1931]. These authors found 3–4 mg./100 ml. by direct precipitation from blood filtrate by means of lime water, but Thomsen [1935] and Voinar & Babkin [1940] have claimed that the precipitate obtained does not consist of oxalate. Thomsen [1935] also criticized the Ce procedure used by Suzuki [1934], but this criticism was answered [Suzuki, 1936] and the method has been in use in a modified form ever since. The purpose of the present paper has been to investigate the Ce procedure used by Kamiya, Noye & Sato [1937] and the Ca procedure of Merz & Maugeri [1931], since these two methods agree in yielding high and unconfirmed results. The ester procedure of Barber & Gallimore [1940] has also been examined.

METHODS

(1) *Investigation of the cerous oxalate method* [Suzuki, 1934; Kamiya *et al.* 1937]. Thomsen [1935], following the procedure of Suzuki [1934], could obtain no precipitate from blood filtrates, and pointed out that $Ce_2(C_2O_4)_3$ is soluble in excess of $CeCl_3$. Suzuki [1936] suggested that Thomsen had overlooked or lost the small precipitate of $Ce_2(C_2O_4)_3$ and insisted that special centrifuge tubes were required. Subsequently, Kamiya *et al.* [1937] suggested $Ce_2(SO_4)_3$ as precipitant since an excess of this substance has less tendency to redissolve $Ce_2(C_2O_4)_3$.

In the present experiments the special centrifuge tubes recommended by Suzuki [1936] were employed. With 2 drops of 0·1% $Ce_2(SO_4)_3$ and 3 ml. of a solution of $Na_2C_2O_4$ equivalent to 1·33 mg. $H_2C_2O_4$/100 ml., no difficulty was encountered in obtaining a crystalline precipitate of $Ce_2(C_2O_4)_3$ at *p*H 6. In 2 hr. 98% of the oxalate was precipitated. This result indicates that an aqueous solution of oxalate of the strength which Suzuki [1934] supposed to be present in blood filtrates yields an easily visible crystalline precipitate, suitable for analysis. However, when the precipitation was attempted under the conditions set out by Izumi [1933], viz. at *p*H 2·3 (colorimetrically, thymol blue) and in the presence of $CCl_3.COOH$, no precipitate could be obtained. In these experiments the number of drops of $Ce_2(SO_4)_3$ was varied from 1 to 4 (0·04–0·16 ml.) and the solutions were left overnight. Since oxalate is not precipitated from aqueous solution under these conditions it is improbable that a precipitate of $Ce_2(C_2O_4)_3$ would separate from a blood filtrate. Experiments showed that in some cases a small amorphous precipitate did appear, but in no case could oxalate be recovered when the precipitate from 10 ml. of blood was subjected to the process of Dodds & Gallimore [1932]. This result therefore supports the contention of Thomsen [1935].

(2) *Investigation of the precipitate obtained with lime water* [Merz & Maugeri, 1931]. If the precipitate consists of CaC_2O_4 it should be possible to convert it into $Et_2C_2O_4$ and to estimate the quantity so esterified by the procedure of Dodds & Gallimore [1932]. The 'oxalate' content of a sample of heparinized human blood was determined, and 4·55 mg./100 ml. were found. The precipitate from 20 ml. of blood (0·91 mg. 'oxalate') was dissolved in a small quantity of HCl and transferred to the Claisen flask of the apparatus used for the distillation of the ester. Only 0·092 mg. oxalate was recovered. It is probable that at least a part of this oxalate arose from the decomposition of ascorbic acid of

the blood filtrate when the latter was made alkaline with lime water. The whole procedure was verified by carrying out a 'blank' experiment in which 1 mg. $H_2C_2O_4$ was added and 1·06 mg. recovered. These results confirm the findings of Thomsen [1935], and of Voinar & Babkin [1940].

(3) *Investigation of the ethyl oxalate procedure* [Barber & Gallimore, 1940]. This method entails a preliminary deproteinization with phosphotungstic acid, evaporation of the filtrate, and precipitation of the oxalate as CaC_2O_4. 10 ml. samples of blood were employed in order to insure the presence of sufficient oxalate to give a clear-cut precipitate. The volume of fluid prior to the addition of lime water was not allowed to exceed 2 ml. The only other modification adopted was the use of an ice-bath in the distillation of the ester, as suggested by Athanasiou & Reinwein [1934].

The most likely sources of error in this method are: (*a*) Loss of $H_2C_2O_4$ by volatilization during the evaporation of the blood filtrate *in vacuo*. (*b*) Incomplete extraction of the $H_2C_2O_4$ with ether. (*c*) Incomplete precipitation by means of lime water. (*d*) Production of $H_2C_2O_4$ from other constituents of the blood during the prolonged and somewhat drastic processes involved in the determination.

The first three possibilities were investigated by carrying out a blank test on the reagents with and without the addition of a quantity of $H_2C_2O_4$ likely to occur in blood. In these tests the amount of phosphotungstic acid was reduced from 4 to 0·2 g., since with blood nearly all the phosphotungstic acid is removed by the proteins. When 0·1 mg. $H_2C_2O_4$ was added to the 'blank' 82% of it was recovered. This recovery is satisfactory. It is probably an advantage to have a small quantity of oxalate present in the reagents, as although the absolute value of the 'blank' is unlikely to be obtained owing to unavoidable losses, such losses occur in both 'blank' and 'test' and the difference, which is the value required, is not likely to be affected.

Nine specimens of normal heparinized human blood were analysed. The values obtained were 0·23, 0·31, 0·40, 0·42, 0·47, 0·51, 0·57, 0·60, 0·77 mg. oxalate/100 ml. with an average result of 0·48 mg./100 ml. (In two cases 0·06 mg. $H_2C_2O_4$ was added to two 10 ml. samples of blood; the recoveries were 84 and 85%.) These figures are in close agreement with those reported by Barber & Gallimore [1940] although the range for normal blood is wider than that (0·4–0·6 mg./100 ml.) given by them.

l-Ascorbic acid was selected as the most probable normal constituent of blood which might yield $H_2C_2O_4$ when subjected to the procedure of Barber & Gallimore [1940]. Its easy conversion into $H_2C_2O_4$ has already been noted by Flaschenträger & Müller [1938] and Jurist & Christiansen [1939], while Scheinkman [1940] found an increased output of oxalate in guinea-pigs after administration of *l*-ascorbic acid. The *l*-ascorbic acid used in the following experiments was supplied by B.D.H., and gave no precipitate when treated with Ca salts at *p*H 5. In three cases 4 mg. of ascorbic acid were added to 10 ml. samples of human blood and the oxalate content was determined. In each case an increase was obtained. The average value of the three specimens prior to addition of *l*-ascorbic acid was 0·50 mg./100 ml. and after the addition rose to 1·5 mg./100 ml. An addition of 40 mg. *l*-ascorbic acid to 100 ml. blood therefore increased the apparent oxalate content by 1·0 mg. If this oxalogenic effect is directly proportional to the quantity of *l*-ascorbic acid present, the quantity of oxalate formed by the 1 mg. of *l*-ascorbic acid present in 100 ml. of normal blood would be 0·025 mg. This value is not of great significance when compared with the normal figure of 0·5 mg./100 ml. but it does indicate that all the $H_2C_2O_4$ found by this method is not necessarily present in the original blood.

DISCUSSION

No evidence could be found in support of the high level of blood oxalate reported by the Japanese group of workers. A negative result of this type is not entirely satisfactory, and it may be argued that the reagents employed in this investigation were not pure. However, only three reagents were used in the $Ce_2(C_2O_4)_3$ experiments. Two of these substances, viz. CCl_3.COOH and NaOH, were of Analar quality, while the $Ce_2(SO_4)_3$ was specially prepared from $Ce(SO_4)_2$. The latter was purified by the procedure of Bowles & Partridge [1937]. Izumi [1933], the originator of the cerium procedure, conducted the precipitation at *p*H 2·3. He investigated the effect of *p*H on the precipitation by mixing 5 ml. buffer solution (acetic acid + acetate or glycine + HCl), 0·5 ml. *N*/10 $Na_2C_2O_4$ and 0·2 ml. 2% $CeCl_3$, and found complete precipitation of oxalate at *p*H 2·3 and upwards. It is evident that the conditions in this experiment have little connexion with those existing in a blood filtrate. Here the concentration of oxalate is many times lower, and the Ce salt is employed in 0·1% solution.

It is unfortunate that our present knowledge of the variation of blood oxalate in health and disease has been acquired mainly by the methods of Merz & Maugeri [1931], of Suzuki [1934], and of Kamiya *et al.* [1937]. The defects inherent in these methods appear to be such that any published work in which they have been employed must be regarded with suspicion.

There appears to be little doubt that the procedure of Barber & Gallimore [1940] gives results of the correct order. However, their figures should be regarded as representing the total oxalate derived from the blood, since the demonstration that their final precipitate consists of CaC_2O_4 does not necessarily imply that this quantity of oxalate is present in the original blood.

SUMMARY

1. No evidence could be obtained to support the contention of Kamiya *et al.* [1937] and Merz & Maugeri [1931] that the oxalate content of blood is 2–4 mg./100 ml.

2. The method of Barber & Gallimore [1940] for the determination of oxalate in blood is confirmed, but the quantity of oxalate involved is too small to permit great accuracy of determination. It is not certain that all the oxalate found by this method pre-exists in the blood.

It is a pleasure to thank Prof. E. C. Dodds for his interest in this work and for his helpful advice.

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A New Technique for the Determination of Phosphorus by the Molybdenum Blue Method

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Numerous methods are available for the colorimetric determination of phosphorus by molybdenum blue. A number of reducing agents have been applied, but no method can be regarded as entirely satisfactory. The methods using $SnCl_2$ have the advantage of high sensitivity, but in general do not obey Beer's Law over a wide range, and the variation in tint at different intensities is sufficient to cause difficulty in matching. Bodansky [1932] corrected for the deviation from Beer's Law in the method of Kuttner & Cohen [1927]. When absolute colour measurement is adopted difficulty is experienced in controlling the colour development and in eliminating variation caused by instability of the colour with time and with changes in room temperature. In methods using other reducing agents these disadvantages are reduced, but the sensitivity is also diminished. The method of Fiske & Subbarow [1925] is probably the most popular and, even though the colour is not quite stable, Allen [1940] has found that a Pulfrich photometer can be used for matching. Probably the only method which gives a stable colour under conditions which are easy to control is that of Zinzadze [1935] using metallic Mo as the reducing agent. A recent review of methods is given by Woods & Mellon [1941].

Arising from an observation that KI in acid solution is a suitable reducing agent for phosphomolybdic acid, a new method has been developed which has none of the above disadvantages and is simple to operate. The range is 1–100 μg. P and, where applicable, it gives a higher degree of accuracy than existing methods. The sensitivity is almost three times that of the Fiske-Subbarow method. Wu [1920] suggested the use of HI as a reducing agent for phosphomolybdic acid and devised a rough test. Berenblum & Chain [1938] used HI in a study of the mechanism of the reduction to Mo blue.

Suitable conditions for reduction to Mo blue and for removal of liberated I_2 were determined. A temperature of 100° was found to be suitable for the development of the colour. The colour intensity increases with the amount of molybdate used, but the 'blank' colour increases in the same manner. The 'blank' can be reduced by increasing the acidity or by using less reducing agent, but if the acidity is too high the colour fades after reaching its maximum intensity at 100°. If too little reducing agent is present the colour is not stable. Suitable concentrations of reagents were ascertained. $Na_2S_2O_3$ cannot be used to remove the liberated I_2 owing to precipitation of S, but Na_2SO_3 was satisfactory. Only a small excess is permissible, otherwise it imparts a yellow colour to the solution. The H_2SO_4 and $(NH_4)_2MoO_4$ must be added separately since a combined reagent gives a high and variable 'blank'. The solution of KI keeps satisfactorily if it contains a small amount of Na_2CO_3 to prevent liberation of free HI.

METHODS

Reagents

(1) *10 N H_2SO_4.*

(2) *2·5% ammonium molybdate solution.* Dissolve 25 g. $(NH_4)_2MoO_4$ in distilled water by warming. Cool and dilute to 1 l. Preserve the solution under liquid paraffin to prevent diffusion into the air of the NH_3 liberated by salt hydrolysis. An aspirator bottle may be used, which has been fitted with a well-greased tap at the base and the inside of which has been coated with a thin film of liquid paraffin, to prevent the solution wetting the sides.

(3) *20% KI solution.* Dissolve 200 g. KI and 5 g. Na_2CO_3 in distilled water and dilute to 1 l. The solution is stable for at least 3 months and probably indefinitely.

(4) *0·5% Na_2SO_3 solution.* Dissolve 1 g. $Na_2SO_3.7H_2O$ in distilled water and dilute to 200 ml. Prepare the solution freshly each day.

(5) *Standard P solution.* Prepare a stock solution containing 1 g. P/l. by dissolving 4·3885 g. pure dry KH_2PO_4 in distilled water, adding 2 ml. concentrated H_2SO_4 as a preservative before diluting to 1 l. To prepare a working standard, dilute 5 ml. of the stock solution to 250 ml. with distilled water. This solution contains 20 μg. P/ml.

Procedure

Transfer a sample containing 1–100 μg. P to a 10 ml. volumetric flask or to a 25 ml. test tube provided with a stopper and marked at levels corresponding to exactly 5 and 10 ml. If the solution is acid or alkaline add phenolphthalein and neutralize with N/10 NaOH or N/10 HCl; then add H_2O to give exactly 5 ml. Add 1 ml. 10 N H_2SO_4, 1 ml. $(NH_4)_2MoO_4$ and 1 ml. KI solution, in the order named and mixing the contents after each addition. Transfer to a briskly boiling water-bath, using an inverted specimen tube to cover the neck of the flask or tube. After exactly 15 min. transfer at once to cold running water. Replace the stopper. After cooling, add Na_2SO_3 from a burette, 0·1 ml. at a time, mixing after each addition, until all the liberated I_2 has been removed and the solution is a pure blue. The end-point is very sharp in daylight or when a daylight lamp is used, but yellow light obscures it somewhat. Then add 0·2 ml. Na_2SO_3 in excess. Make the volume to 10 ml. with H_2O and mix. Match the colour by means of a photoelectric absorptiometer or a visual colorimeter.

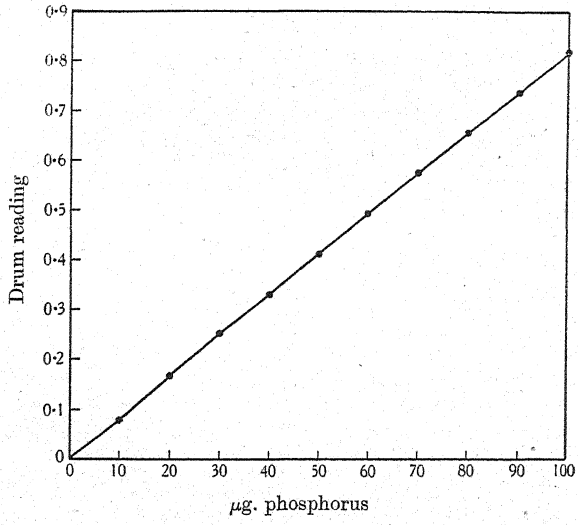

Fig. 1. Typical calibration curve, using the Spekker photoelectric absorptiometer.

If a Spekker photoelectric absorptiometer is used it is necessary to prepare a 'blank' from distilled water treating it in the same way as the test solution. Place red glass filters (no. 1) in front of the two photocells and a 1 cm. glass cell containing the test solution in front of the right photocell. Set the drum to zero and bring the galvanometer reading to zero by adjusting the shutter aperture in front of the left photocell. Then place a 1 cm. glass cell containing the 'blank' in front of the right photocell and again bring the galvanometer reading to zero by rotating the drum, while keeping the shutter aperture constant. Record the drum reading. Switch off the lamp in the interval between each of a series of matchings to prevent fatigue in the photocells, but not in the interval between the two operations described above which comprise a single matching. To obtain a calibration curve from which the amount of P in the test solution can be read match a series of standards against the 'blank' and plot the drum readings against the corresponding amounts of P. The curve should be prepared by each worker for his own instrument and checked periodically. Colours giving drum readings greater than about 1 are too intense for accurate results to be obtained and, if 1 cm. glass cells are used, only the range 1–30 μg. P can be covered.

To cover the range 30–100 μg. P the colour must be diluted to one-third of its intensity if 1 cm. glass cells are to be used. After developing the colour remove the liberated I_2 with 0·5% Na_2SO_3 and add 0·5 ml. in excess. Dilute to 30 ml. instead of 10 ml. and match against a 'blank' prepared in the same way. A calibration curve using this procedure is shown in Fig. 1.

If a visual colorimeter is used for matching dilution to 30 ml. is not required. It is necessary, however, to correct for the 'blank' and for this reason a 'blank' and a standard containing 1 μg. P should be prepared. Match the 'blank' against the standard and calculate the amount of P to which its colour is equivalent as follows:

$$c = \frac{b}{(a-b)},$$

where c = μg. P to which 'blank' is equivalent; a = length of column of 'blank'; b = length of column of 1 μg. standard which matches a.

Make the following correction for the 'blank' when calculating the result for the test:

$$x = \frac{b\,(y+c)}{a} - c,$$

where x = μg. P present in test solution; y = μg. P present in standard; c = μg. P to which 'blank' is equivalent; a = length of column of test solution; b = length of column of standard which matches a.

DISCUSSION

Order of accuracy of the method

The colour is stable, no difference in intensity being detectable when standards were kept up to 24 hr.

in stoppered test tubes or when the temperature was varied from 12 to 32°. After keeping for some days the excess of Na_2SO_3 gradually becomes oxidized and further I_2 is liberated thus altering the colour. Small variations in the time of development of the colour at 100° have little effect on the intensity. The variations when the time was increased or decreased by 5 min. are shown in Table 1. The average error per min. of deviation from the standard time of 15 min. is 0·3 %.

Table 1. *Effect of time of heating on the development of the colour*

Time of heating min.	P present μg.	P found μg.	Error %
10	10	9·7	−3·0
10	50	49·2	−1·6
10	100	98·0	−2·0
15	10	10·0	Nil
15	50	50·0	Nil
15	100	100·0	Nil
20	10	9·8	−2·0
20	50	50·0	Nil
20	100	101·0	+1·0

When a Spekker photoelectric absorptiometer is used for matching, results accurate to within 2 % can be obtained for the range 3–100 μg. P. With special care the error may be reduced still further. Fig. 1 shows that the relationship between drum reading and amount of P is very nearly linear. Deviations from strict linearity are attributable to the photocells themselves as well as to the colour.

When a visual colorimeter is used and a correction is made for the 'blank', Beer's Law is obeyed closely enough for reasonable accuracy to be obtained even when the test solution is widely different from the standard. A high degree of accuracy can be assured if each test solution is matched against a standard which does not differ from it by more than about 30 %. With less than 5 μg. P the colours are too weak for accurate matching, and the deviation from Beer's Law is greatest in this region. Results for a series of standards containing widely different amounts of P are shown in Table 2. The colours were matched by means of a Klett colorimeter.

Table 2. *Order of accuracy with a visual colorimeter*

P present μg.		Relative lengths of columns		P found μg.	Error %
Test	Standard	Test	Standard	Test	
'Blank'	1	40·0	25·0	1·7*	—
10	50	30·0	6·7	9·84	−1·6
10	100	30·0	3·5	10·16	+1·6
50	10	6·7	30·0	50·7	+1·4
50	100	10·0	5·1	50·2	+0·4
100	10	3·5	30·0	98·6	−1·4
100	50	5·1	10·0	99·7	−0·3

* This value was used to correct for the 'blank'.

Application of the method

Salts likely to be present in solutions to be tested were included in standards in various concentrations before development of the colour. The maximum amount which may be present in the sample without affecting the colour was determined in each case (Table 3).

Table 3. *Maximum amounts of various salts which may be present without affecting the result*

Salt	Maximum amount permissible (mg.)	
$FeCl_3$	0·1	Fe^{+++}
$CaCl_2$	2	Ca^{++}
$MgCl_2$	40	Mg^{++}
NaCl	100	Cl^-
Na_2SO_4	10	$SO_4^=$
Na acetate	5	CH_3COO^-
Na trichloracetate	100	CCl_3COO^-
Na citrate	5	$C_6H_5O_7^{\equiv}$

These results were obtained when a single salt was present and do not necessarily apply to mixtures of salts. Thus, a mixture consisting of the maximum amounts of all these salts was found to reduce the colour intensity by approximately 4 %. It is therefore advisable, before applying the method to any particular type of solution, to make certain that the recovery of added P is complete, even though the amounts of salts present do not exceed the limits given in Table 3.

Not more than a trace of Cu^{++} may be present, owing to precipitation of CuI with liberation of I_2. The maximum amount which may be present was found to be 200 μg. Cu^{++}.

Since As and Si produce a blue colour in the same way as P, the maximum amounts which may be present without affecting the test were determined and are for Na_3AsO_4 1 μg. As, and for waterglass 10 μg. SiO_2.

The method is suitable for the analysis of HCl extracts of residues from dry ashing or Na_2CO_3 fusion. It cannot be used to distinguish between inorganic and organic P compounds. Several organic P compounds were found to be hydrolysed under the conditions of the test.

SUMMARY

A new technique has been developed for the determination of phosphorus by molybdenum blue, using potassium iodide as reducing agent. The range of the method is 1–100 μg. P. The colour is stable and obeys Beer's Law closely if a correction is made for the faint colour present in the 'blank'.

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Oxidizing Enzymes and Vitamin C in Tomatoes

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Sixteen years ago Willimott & Wokes [1926 *a*, *b*, *c*] studied the occurrence of oxidizing enzymes and vitamin C in the peel and juice of citrus fruits. The enzymes were found to be present in highest concentration in the rind. When lemon rind was being assayed for vitamin C, in order to avoid destruction of the vitamin by these enzymes, the rind was cut into small pieces after removal, weighed, and mixed with 90% ethanol to prepare a tincture for administration to guinea-pigs. This tincture failed to prevent scurvy when administered as the sole source of vitamin C in daily doses equivalent to 0·5–1 g. of the rind, although the onset of symptoms was slower than in negative controls, so lemon rind was assumed to contain only slight amounts of the vitamin as compared with the juice, which has a considerable amount.

Later, when a satisfactory chemical test for vitamin C had become available, this was applied by Bacharach, Cook & Smith [1934] to orange and lemon rinds. Orange rind, which Willimott & Wokes [1926*c*] had reported to contain only moderate amounts of the vitamin, was found to contain a concentration of vitamin C several times that of the juice, whilst lemon rind also contained more than lemon juice. Bacharach *et al.* [1934] suggested that the discrepancy between their results and those of Willimott & Wokes might be due to loss of vitamin during preparation of the tincture, but made no reference to the oxidizing enzymes. Willimott & Wokes had drawn attention to these enzymes, but assumed that the use of 90% ethanol, in which the enzymes would not be soluble, would prevent their destroying the vitamin. Only within the last few months has it been realized that when these enzymes are set free by mincing the tissues a considerable amount of the vitamin may be destroyed in a few minutes.

In the interval between the appearance of the papers of Willimott & Wokes [1926 *a*, *b*, *c*] and that of Bacharach *et al.* [1934] Szent-Gyorgyi [1931] showed that his 'hexuronic acid' was rapidly destroyed by an oxidizing enzyme when the inner leaves of cabbage were minced. Qualitative tests indicated that most of the 'hexuronic' acid had disappeared within 5 min. of completion of mincing. The enzyme was named hexoxidase because it attacked hexuronic acid, but since the latter is now known as ascorbic acid the enzyme is now known as ascorbic acid oxidase.

Kohman, Eddy & Gurin [1931] reported that minced carrots lost much of their antiscorbutic activity on standing in air for 1 hr., but biological assays did not prove suitable for investigating this problem. Zilva [1934], using both biological and chemical methods, showed that apple juice contains an enzyme which destroys the vitamin C in lemon juice, approximately 84% being lost at *p*H 4·4 and 57% at *p*H 3·0, both in 6 hr. at room temperature, and that, as might have been deduced from the findings of Willimott & Wokes, the juice is a less potent source of the enzyme than the peel. However, during the following 8 years most workers on this enzyme neglected fruit and concentrated on vegetables. Thus Ahmad [1935] showed by chemical assays that 11% of the vitamin C in karela was lost in 10 min. and 17% in 20 min. when this common Indian vegetable was shredded. McHenry & Graham [1935] obtained similar results with shredded turnips, parsnips and cauliflower. Tauber, Kleiner & Mishkind [1935] found the enzyme in *Cucurbita maxima* and named it ascorbic acid oxidase. Hopkins & Morgan [1936] extracted the enzyme from cauliflower florets and showed that the enzyme extract would destroy about 70% of added ascorbic acid in 5 min. at *p*H 6 and 18°. It was apparent from their results that if the ratio of enzyme to ascorbic acid had been similar to that which occurs naturally even more rapid destruction of the vitamin would have occurred. Kertesz, Dearborn & Mack [1936] showed that when cabbage was ground with an equal weight of water it lost about 97% of its ascorbic acid content in 1 hr. and reported similar results with other vegetables. Johnson & Zilva [1937] expressed the juice from frozen cabbage, cauliflower, cucumber and marrow but found that the destruction in 1 hr. of ascorbic acid added to cauliflower juice was only 33% at *p*H 5 and 35% at *p*H 7, much slower than the rate found by Hopkins & Morgan [1936]. They confirmed Hopkins & Morgan's findings, however, that the optimum *p*H for the enzyme is about 6, and showed that at *p*H 3·0 the activity was very slight, but at *p*H 9·0 still fairly pronounced. Stone [1937] minced vegetables containing vitamin C, and showed that in cabbage juice a loss of 98% of free ascorbic acid and about 63% of total ascorbic acid occurred in 5 min. Like Kertesz *et al.* [1936] he found that the rate of destruction in spinach was much slower. Lampitt & Baker [1942] observed that when orange

peel was minced and allowed to stand, 17% of the ascorbic acid was destroyed in 3 hr. and over 90% in 22 hr. Similar results were obtained with cabbage. The destruction of the vitamin was attributed to an oxidase. Pyke [1942] showed that the rate of destruction was increased by dividing the tissue more finely and thus liberating the enzyme sooner. In finely shredded cabbage as much as 32% of the vitamin was destroyed in 5 min., but in swedes the rate of destruction was lower. Lampitt, Baker & Parkinson [1942] confirmed and extended Pyke's results, finding a 40% loss in 1 min. and a 60% loss in 7 min. Wokes & Organ [1942] found similar losses in minced green tomatoes.

EXPERIMENTAL

Ascorbic acid oxidase appears to be present in highest concentration in green vegetables and in the skin of fruits. Ebihara [1938] reached the same conclusion. We selected tomatoes for our experiments, obtaining them from three sources: King's Langley (outdoor), Elstree (greenhouse), Wilmslow, Cheshire (outdoor and greenhouse). Vitamin C was estimated by the dye micro-titration method as developed by Harris & Olliver [1942], using precautions described below. Some results on coloured solutions were checked by the potentiometric method of Harris, Mapson & Wang [1942]. Tests for oxidizing enzymes were applied as described by Willimott & Wokes [1926*b*] both to the tissues themselves, and to aqueous extracts prepared by grinding the tissues in a glass mortar with a special fine grade of quartz sand made by Mr A. L. Curtis of Chatteris. *p*H determinations were made with the glass electrode.

Oxidizing enzymes and p*H*

In unripe tomatoes oxidizing enzymes occur in the highest concentration in the skin; the flesh and seeds contain less, and the juice very little. In ripe tomatoes the concentrations appear to be similar to those in unripe tomatoes, but slightly lower.

The *p*H of the skin of unripe tomatoes was found to be high, ranging from 8·5 to 9·6 in samples examined within a few days of picking. When green tomatoes are kept for several weeks the *p*H of the skin may, under certain conditions, fall slightly, but if ripening proceeds normally the skin seems always to remain alkaline. In making *p*H determinations on the skin of ripe tomatoes it is important to remove all the flesh, which is acid.

The juice of tomatoes appears always to be acid. That of unripe tomatoes ranged from *p*H 3·9 to 5·0 (average 4·4), and of ripe tomatoes from *p*H 4·2 to 4·5. In the flesh the reaction changes from alkaline to acid during ripening. In firm green unripe tomatoes the *p*H of the flesh ranges from 8·4 to 9·0. When such tomatoes are ripened after picking either in the dark or in the light the *p*H gradually falls to between 4·6 and 5·5, a range similar to that of the flesh of tomatoes ripened on the plant.

These findings suggested that the rate of destruction of vitamin C by ascorbic acid oxidase would be most rapid in the skin, where the concentration of oxidase is highest and the *p*H most favourable, and slowest in the juice, where the reverse conditions prevail When whole tomatoes are minced the *p*H of the mince depends largely on that of the flesh. Since the flesh is alkaline in unripe, and acid in ripe tomatoes, the rate of destruction of the vitamin in minced whole tomatoes should be more rapid in the unripe. These suppositions have been verified.

Rate of destruction of ascorbic acid in different parts of the tomato

The precautions adopted are as follows: the skin was removed in small pieces with a stainless steel knife from four green tomatoes of similar weight and from the same source. Each piece was immediately either immersed in a freshly prepared solution of HPO_3 containing 25 g./100 ml. in a tared weighing bottle or alternately in one of two empty weighing bottles labelled respectively 'control' and 'experimental'. The three bottles were weighed to determine the amount of skin in each; the skin from one of the empty weighing bottles was transferred quantitatively to a glass mortar with quartz sand and distilled water. At a given time grinding was begun, and it was continued for the required time, say 5 min., further quartz and water being added as necessary. Exactly 5 min. after grinding commenced HPO_3 solution was added to stop the action of the oxidase; the contents of the mortar were pressed through muslin; the mass was returned to the mortar and extracted twice more with acid and water, and the combined extracts and washings made up to 100 ml. At the start of the grinding HPO_3 solution had been added to the control. The contents of the control bottle and of the bottle which initially contained acid were extracted in the same way, each being made up to 50 ml. and the ascorbic acid content determined. The concentration of ascorbic acid in the skin of the control was assumed to be the same as that in the experimental skin when grinding commenced. This procedure is necessary because with skin an appreciable amount of ascorbic acid may be lost merely on standing, through the action of oxidase liberated when the skin is removed. In the above experiment 19% of the initial ascorbic acid was lost in the skin whilst standing for 30 min. Of the remainder, 98% of the free ascorbic acid and 95% of the total ascorbic acid were destroyed during the 5 min. grinding.

There are several possible sources of error in this technique, but consistent results were obtained in a series of 26 experiments, summarized in Table 1. The rate of de-

Table 1. *Rate of destruction of vitamin C by oxidase liberated on grinding different parts of tomatoes with quartz sand*

Period of grinding min.	Skin		Flesh		Whole	
	Unripe %	Ripe %	Unripe %	Ripe %	Unripe %	Ripe %
1	67	47, 50	—	—	—	—
2	69	76	38	26	26, 28	—
3	84	—	—	—	—	6
5	98	99	—	—	—	27
10	—	—	64	48	—	—
12	—	—	Juice		—	29
15	—	—	64	—	95	33
20	—	—	—	19	—	—
30	—	—	—	—	—	37
60	—	—	—	—	—	45
120	—	—	—	14	—	71
20 hr.	—	—	—	—	—	95

The results represent percentage of vitamin C destroyed by grinding for the given time.

All results obtained on the juice and whole tomato included seeds except the 120 min. sample of ripe juice.

struction was most rapid in the skin and slowest in the juice. When the seeds were removed from the juice before grinding the rate of destruction of the vitamin was still further reduced because of the removal of the seed oxidase. When whole tomatoes were ground with quartz sand or minced, the rate of destruction was more rapid in unripe tomatoes, and intermediate between that for the skin and that for the juice. Moreover, when green tomatoes were sliced the rate of destruction of the vitamin decreased as the slices were cut coarser [Wokes & Organ, 1942]. All these findings suggest that the rate of destruction of the vitamin depends on the concentration of oxidase in the tissues and the rate at which this is liberated by mechanical disruption of the cells.

When weighed amounts of skin were ground with quartz and a known amount of ascorbic acid in phthalate buffer at *p*H 4 the rate of destruction of the ascorbic acid became slower as the ratio of oxidase to ascorbic acid was decreased. When the relation between the oxidase and the ascorbic acid was such as would occur when the skin of a tomato was ground with the whole of the flesh and juice, the rate of destruction was similar to that found when grinding whole ripe tomatoes (e.g. 20% destroyed in 5 min., 45% in 30 min.). This was further confirmation of the validity of the above theory.

Sampling of tomatoes

If the pieces of tomato are too large and skin is present, there may be destruction of the vitamin inside the tissue before the acid can reach and inactivate the enzyme. We used samples of 3–10 g. for whole tomatoes or their flesh, and 0·5–3 g. for the skin. These samples were cut up into small pieces under HPO_3 solution, using a stainless steel knife, and then ground with the quartz sand. Since only a portion of the tomato is used for each assay this raises the question of sampling. As will be seen later, the concentration of vitamin C may vary by several hundred % in different parts of the same tomato. The accuracy of our sampling procedure was tested by analysis of replicate samples, and determining the percentage standard deviation or coefficient of variation of a single assay result. The average results thus obtained on over a hundred samples are summarized in Table 2. Apart from the incubated tomatoes, the results from which are subject to special errors to be considered later, these results indicate that in sampling whole tomatoes our average coefficient of variation for the mean of two assays was about 4. When comparing results on the same tomato we therefore considered a difference of 8% significant ($p=0·95$). Very little information is available regarding the sampling error when estimating vitamin C. Olliver [1938] quotes results on duplicate samples of strawberries giving coefficients of variation of 6·1 for unripe and 2·9 for ripe red fruit, which are of the same order as our own sampling errors for tomatoes.

Table 2. *Sampling errors in vitamin C estimations on tomatoes*

Part of fruit assayed	Coefficient of variation for the estimation of vitamin C in a single sample		
	Unripe	Ripe	Incubated
Whole	5·8	5·1	8·8
Skin	3·1	3·6	7·2
Flesh and juice	8·2	7·8	—

We also considered how much of the so-called sampling errors might be due to titration errors. At least four titrations were done on each sample, the end-point usually being checked by two observers. From the results of many hundreds of titrations we concluded that differences between two assay results due to titration errors would be not more than 2%. When titrating extracts of ripe tomato skin we removed the pink colour by centrifuging. The visual results were also checked by parallel determinations on different samples by the potentiometric method of Harris *et al.* [1942], which gave results agreeing closely with visual results, the average coefficient of variation between the two methods being 1·7 in assays on 12 samples.

RESULTS

Concentration of vitamin C in different parts of the tomato

Our results (Tables 3 and 4) for the estimation of ascorbic acid in the different parts of the flesh, and

Table 3. *Vitamin C concentration in different parts of ripe tomatoes*

Wt. g.	Ascorbic acid (mg./100 g.)				Distribution ratio (skin/whole)
	Whole	Skin	Flesh	Juice	
35·4	13·7	37·9	—	—	2·76
36·2	15·2	30·0	—	—	1·97
40·6	17·6	30·1	14·9	10·7	1·70
44·8	17·9	62·4*	—	—	—
55·9	17·3	25·6	—	—	1·48
56·9	16·0	36·2	11·0	16·9	2·26
61·5	19·2	42·6	—	—	2·22
63·0	19·5	42·2	—	—	2·16
76·6	18·7	42·7	14·1	12·6	2·28
83·4	—	29·4	—	18·2	—
Mean	16·9	35·2	13·3	14·6	2·10
S.D. of mean	1·9	6·6	—	—	0·39
Coefficient of variation	11·5	18·7	—	—	18·6

* This skin was cut off more finely than usual, and the result is not included in the mean.

Table 4. *Vitamin C concentration in different parts of unripe tomatoes*

Wt. g.	Ascorbic acid (mg./100 g.)				Distribution ratio (skin/whole)
	Whole	Skin	Flesh	Juice	
6·9	12·0	23·9	—	—	1·99
7·8	12·9	20·2	—	—	1·58
30·5	10·0	18·3	11·9	8·5	1·83
52·2	23·4	50·0	—	—	2·14
53·3	13·4	23·1	—	—	1·72
58·2	14·9	23·0	—	—	1·54
58·6	15·5	33·9	8·3	17·7	2·16
59·0	18·1	34·0	—	—	1·88
Mean	15·0	28·3	10·1	13·1	1·86
S.D. of mean	4·1	10·5	—	—	0·23
Coefficient of variation	27·3	37·1	—	—	12·4

in the juice, of ripe and of unripe tomatoes, are, we believe, on the basis of our experiments described above, free from errors resulting from oxidase destruction of the vitamins during the separation of the different tissues.

There were wide variations from one tomato to another and the coefficients of variation were much greater than those given in Table 2, showing that the variation in vitamin C content of different tomatoes was not due to sampling errors. Nevertheless, in any given tomato the concentration of vitamin C in the skin was always definitely greater than that in the whole fruit. The distribution ratio (ratio of concentration in skin to that in the whole fruit) ranged from 1·48 to 2·76 (mean 2·10) in ripe tomatoes and from 1·54 to 2·16 (mean 1·86) in unripe tomatoes. The coefficients of variation showed that in unripe tomatoes there was much less variation in this distribution ratio for the skin than in the individual figures for skin and for whole fruit, showing that the factors affecting the vitamin C content of the skin also affected that of the whole fruit. Our data (Table 3) on ripe tomatoes did not show this so clearly, possibly because of variations in the conditions of ripening, of which more will be said later.

Only a few samples of flesh and juice were examined. The vitamin C concentration in these is always much less than in the skin. The mean distribution ratios did not show any significant difference in vitamin C concentration between the flesh, juice or whole fruit. There was also no significant difference between the vitamin C concentrations in different parts of the flesh. On the other hand, the seeds both of ripe and unripe tomatoes contain a much lower concentration of the vitamin than the whole fruit. We were not able to estimate the vitamin C accurately in the seeds of a single tomato, the estimation of which is required in order to calculate individual distribution ratios, but data obtained on pooled samples from a number of tomatoes indicated that the mean distribution ratio for seeds is about 0·2. As the seeds form only about 1 % of the total weight of the fruit their contribution to its vitamin C value is insignificant.

Table 5. *Mean distribution ratios for vitamin C concentrations in different parts of tomatoes*

	Wt. of part % of whole	Distribution ratios	
		Unripe	Ripe
Skin	3–4	1·86	2·10
Flesh	95–96	0·87	0·76
Juice		1·00	0·78
Seeds	1	0·2	0·2

Distribution ratio for any given part represents concentration of vitamin C in that part divided by the concentration in the whole tomato from which the part was taken.

In Table 5 are given the mean distribution ratios for vitamin C in different parts of the tomato. These show a steady drop in the concentration of the vitamin as one proceeds from the skin inwards. The concentration in the skin is 2 or 3 times that in the flesh and juice, and about 10 times that in the seeds. This applies equally to unripe and ripe fruit.

Factors affecting vitamin C content of tomatoes

(*a*) *Weight and polyploidy.* Since the concentration of vitamin C in the skin is 2 or 3 times that in the flesh and juice, and since the proportion of skin to total weight of fruit decreases as tomatoes become larger, the concentration in the whole fruit should diminish as tomatoes became larger. This was predicted by McHenry & Graham [1935] who suggested that it would explain why Sansome & Zilva [1933] had found a higher concentration of vitamin C in tetraploids than in diploids, the former being on the average smaller than the latter. Sansome & Zilva [1936] gave further data supporting their previous findings in regard to polyploidy and its effect on the vitamin C content, and did not accept the suggestion of McHenry & Graham that the weight of the tomatoes might be a deciding factor. None of these workers, however, gave the data for the relative concentrations of the vitamin in the skin and other parts of the tomato which were essential to test the validity of the argument. Rudra [1936] found 57 mg./100 g. in the skin of Indian tomatoes, but as the flesh of these tomatoes contained 39 mg./100 g., much more than was found in British tomatoes either by Sansome & Zilva or by ourselves, Rudra's results are not applicable to the problem of polyploidy.

We found that in tomatoes weighing not less than 20 g. the skin consisted of less than 4, and usually less than 3 %, of the total weight. If the concentration of vitamin C in the skin is 3 times that in the flesh or juice, an increase from 3 to 6 % in the proportion of skin would cause an increase of only 6 % in the concentration of the vitamin in the whole fruit (i.e. the concentration in the latter case would be 106 % of that for the former).

The difference in vitamin concentration between diploids and tetraploids observed by Sansome & Zilva [1933] was about 68 %, i.e. average content for diploids 22 mg./100 g. and for tetraploids 37 mg./100 g. This was a much greater value than the 6 % which we calculate would be produced by doubling the proportion of skin. Actually the proportion of skin in Sansome & Zilva's tetraploids (mean wt. 13 g.) would be less than twice that in their diploids (mean wt. 42 g.). We therefore consider that the variation in weight in Sansome & Zilva's tomatoes made no significant difference to their conclusions regarding polyploidy.

The results of estimations on 29 unripe and 20 ripe diploid tomatoes all grown under the same conditions are summarized in Table 6, and show that in unripe tomatoes

Table 6. *Vitamin C concentration in tomatoes of different weights*

No. of samples	Range in wt. g.	Mean wt. g.	Vitamin C (mg./100 g.) Range	Mean	S.D. of mean
		Unripe			
5	0·3–11	6·6	10·6–33·3	25·0	4·0
5	18 –26	22	12·6–28·8	21·2	2·8
9	30 –47	39	10·5–20·7	15·1	1·5
10	50 –87	62	13·1–27·3	17·7	
		Ripe			
20	36 –83	52	11·2–21·4	16·7	0·9

weighing 0·3–11 g. the average concentration of vitamin C was about 34% higher than in unripe tomatoes weighing 30–87 g. However, the difference was scarcely significant because of the wide variation in vitamin C content between tomatoes of similar weights. Hence, in order to obtain a conclusive answer regarding the effect of polyploidy on vitamin C concentration it will be necessary to control other factors affecting the vitamin content in order to reduce the variations we have disclosed. The importance of these factors was recognized by Sansome & Zilva [1936] in a further examination of the problem. We might add that when examining another series of diploid tomatoes grown in a greenhouse at Bushey we found no significant difference in vitamin C concentration for a weight range of 7–59 g., and we are inclined to think that the difference of 34% observed in our King's Langley tomatoes was rather above the average.

(*b*) *Conditions of ripening.* We were not able to find a significant difference in mean vitamin C concentration between unripe and ripe tomatoes. Thus 21 unripe tomatoes weighing 18–87 g. (average 41·2 g.) had an average content of 17·1 mg./100 g. as compared with an average of 17·5 in 15 ripe tomatoes weighing 41–77 g. and averaging 51·7 g. These tomatoes were all grown under the same conditions. Green tomatoes collected from the same plant growing out of doors at King's Langley were divided into three balanced sets of ten. One set was ripened in sunlight, the second in the dark at room temperature, and the third in an incubator at 37°. As the tomatoes became ripe they were assayed for their vitamin C content with the precautions suggested above. Table 7 shows that the vitamin C content of those ripened in sunshine averaged 17·7 mg./100 g. (similar to tomatoes ripened on the plant); that those ripened in the dark averaged only 12·9, and that those ripened in the incubator averaged only 8·5. Also the tomatoes ripened in sunshine tended to increase in vitamin content as ripening was prolonged, while those in the dark showed a gradual loss of vitamin after the first few weeks, especially at 37°.

Table 7. *Effect of sunlight and heat on vitamin C content of ripening tomatoes*

Period of ripening days	mg. ascorbic acid/100 g. tomato Conditions of ripening: In 'sunlight' at room temp.	In dark at room temp.	In dark at 37°
2	—	—	11·1
6	—	—	12·7
8	11·4	—	10·0
11	—	12·7	—
14	16·0	17·6	13·4
20	18·7	14·6	8·4
20	—	17·3	—
22	—	15·4	—
22	17·6	11·2	—
27	17·9	8·8	6·2
28	19·2	12·1	—
31	14·9	—	6·5
32	13·4	—	6·4
35	19·5	10·3	—
37	—	9·4	—
42	28·5	—	2·3
Mean	17·7	12·9	8·5

Results are all calculated on initial weights of tomatoes (i.e. at beginning of ripening period). During this period there were gradual losses in weight, especially in the tomatoes ripened at 37°, in which the loss reached 30% after 4 weeks.

Since these results indicated that exposure to sunlight affects the vitamin C content we tried the effect of exposing tomatoes to ultra-violet light during ripening. Green tomatoes from King's Langley were irradiated daily by exposure to a mercury vapour lamp at a distance of 1 ft. This provided no appreciable amount of radiation below 250 mμ and we do not think it was destroying any of the vitamin. In order to diminish the heating effect, the lamp was switched off for a few minutes every 10 or 15 min. The total amount of irradiation was 1 hr. on the first day, gradually increasing to 2 hr. on subsequent days. Unfortunately the irradiation did not produce normal ripening, the skins becoming deeply wrinkled and the flesh being softer than usual. The mean vitamin C content in the tomatoes after 4–7 days' irradiation was 16·9 mg./100 g., no evidence being obtained of an increase in vitamin content. Brown & Moser [1941] found the concentration of vitamin C in tomatoes grown in greenhouses to be only half that of tomatoes grown out of doors. Our data support these findings: five grown in the open had an average content of 15·2 mg./100 g. as compared with 12·6 for five grown in a greenhouse. The number of samples examined was, however, not large enough to make this difference significant.

While exposure to sunlight may increase the vitamin C content of tomatoes, we attribute some at least of the low results we obtained on tomatoes kept in the dark for 5–7 weeks to enzyme action. These tomatoes showed a softening of the flesh immediately beneath the skin which probably allowed the ascorbic acid oxidase to gain access to the juice. Other workers have reported increases in vitamin C content when tomatoes were ripened to a soft condition in sunlight, but our degree of softening was probably greater. A tomato which had been ripening in the sunlight for 7 weeks had a slight bruise extending from just beneath the skin towards the centre. When taking

samples we avoided the bruised part, and our two samples gave results of 27·4 and 29·5 mg./100 g. Two further samples taken from parts in contact with the bruise, but not including it, gave results of 11·3 and 14·5. In our opinion these results indicate the possibility of a rapid loss of vitamin C in bruised tomatoes by the penetration of the oxidase into the juice. The tomato appeared perfectly normal apart from the bruise.

DISCUSSION

Although tomatoes are not the richest natural source of vitamin C they possess advantages over the green leafy vegetables and small fruits such as blackcurrants and rose hips in studies relating to this vitamin. One of the most pressing problems, as pointed out by Bacharach *et al.* [1934], is to explain the wide variations in vitamin C content found in different samples of a given fruit or vegetable. Bacharach *et al.* [1934] worked with citrus fruits, big enough to permit the different tissues to be examined separately, so that the distribution of the vitamin could be studied. If Bacharach *et al.* [1934] had correlated their results with the previous findings of Willimott & Wokes on *p*H and oxidases in citrus fruits, they would not only have explained the low results obtained by Willimott & Wokes for vitamin C in citrus peels, but also have provided information of general interest on vitamin C in relation to ascorbic acid oxidase. During the present shortage of citrus fruits in this country tomatoes form an excellent alternative for investigation. We were able to study the occurrence of vitamin C and ascorbic acid oxidase in different tissues, and to evaluate errors due to sampling and other causes. Lack of such knowledge must affect the validity of conclusions drawn by previous workers.

In the short season during which home-grown tomatoes are available conclusive evidence could not be obtained on all the points we investigated. The effect of weight and polyploidy on vitamin C concentration was left uncertain. Our failure to confirm the findings of Brown & Moser [1941] that tomatoes ripened out of doors contain twice as much vitamin C as those ripened in a greenhouse was perhaps due to the vagaries of the British climate, for we have little doubt that our out-of-door ripened tomatoes received much less sunshine than those of Brown & Moser. This lack of sunshine may explain why our tomatoes contained less vitamin C than other workers have found in tomatoes grown in warmer climates. The only other results we could find for British grown tomatoes were those of Sansome & Zilva [1933], whose average figure of 22 mg./100 g. for diploids was higher than our figures of 15–18, but lower than McHenry's [1935] figures of 18–36 for Canadian tomatoes, and considerably lower than the figure of 39 obtained by Rudra [1936] for Indian tomatoes.

In rose hips Melville & Pyke [1942] found very much higher concentrations of vitamin C in species native to the north of England and Scotland than in those of the south of England. Their findings, which were obtained on a large number of small samples, have been confirmed on large-scale samples comprising several tons of hips by Wokes, Johnson, Duncan, Organ & Jacoby [1942], and in other unpublished results. Sabalitschka & Priem [1941] reported that hips growing on the north side of a bush contained more vitamin C than those growing on the south side of the same bush. All these findings suggest that the effect of sunshine on vitamin C formation in plants needs to be considered.

The effect of oxidizing enzymes on vitamin C also merits further investigation. Our results show that if this is not allowed for there may be serious losses of the vitamin. We have provided some evidence that when tomatoes are ripened and stored under conditions favouring enzyme action the oxidase may destroy much of the vitamin. On the other hand, the highest concentrations of vitamin C are usually found in tissues containing the largest amounts of oxidase, suggesting that under certain conditions the action of the oxidase may be reversed, and synthesis of vitamin C take place. In this connexion our findings on the occurrence of oxidase in tomato seeds are of interest, since the seeds when examined contained no significant amount of vitamin C, but the latter could be formed by germinating the seeds in the dark.

In our opinion exposure to sunshine and contact with ascorbic acid oxidase will prove to be of much greater importance than factors such as polyploidy in determining the vitamin C value of foods.

SUMMARY

1. Oxidizing enzymes occur in highest concentration in the skin of both ripe and unripe tomatoes. The flesh and seeds contain less enzyme and the juice practically none.

2. The skin is markedly alkaline (*p*H 8·0–9·6) in both ripe and unripe tomatoes. The reaction of the flesh changes from the alkaline (*p*H 8·4–9·0) to the acid (*p*H 4·6–5·5) side during ripening. The juice appears always to be acid (*p*H 3·9–5·0).

3. When oxidase is set free by mechanical disruption of the tissues it destroys vitamin C. The rate of destruction is most rapid in the unripe skin (e.g. 67 % destroyed in 1 min.), less rapid in the flesh and slowest in the juice, paralleling the oxidase concentration and *p*H in these tissues.

4. The distribution of vitamin C usually runs parallel with the oxidase concentration, being 2 or 3 times higher in the skin than in the juice or flesh. The higher concentration of oxidase but lower concentration of vitamin C in the seeds may be related

to the fact that vitamin C can be produced in the seeds by germination.

5. Because of the variation in vitamin C concentration in different tissues and the action of oxidase during dissection, the sampling error is considerably higher than the normal titration error, and if this is not taken into account it may seriously affect the results.

6. Very small tomatoes may, because of the higher proportion of skin, contain higher concentrations of vitamin C, but the differences found in tomatoes grown under the same conditions were not large enough to discredit the findings of Sansome & Zilva on polyploidy, as McHenry & Graham had suggested.

7. Exposure to sunshine during ripening may affect the vitamin C content of tomatoes, but the latter is also affected by the oxidase, which may destroy a considerable amount of the vitamin if ripening takes place under conditions favouring oxidase action.

We are indebted to Sir Harry Hague and Mr H. H. Jones for samples of tomatoes grown under known conditions, to Dr C. D. Darlington for kindly examining seeds of our tomatoes for polyploidy, and to Miss M. Payne for assistance in preparing the extracts.

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The Anti-streptococcal Action of Iodinin. Naphthaquinones and Anthraquinones as its Main Natural Antagonists

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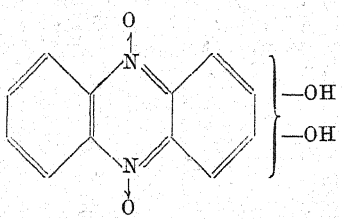
Chromobacterium iodinum was isolated and so named owing to the purple, bronze-glinting pigment which covers its colonies on suitable solid media [Davis, 1939]. This pigment, here termed 'iodinin', was found to inhibit the growth of certain other bacteria [McIlwain, 1941*a*]; and such may be its natural function. The organisms so inhibited include pathogens, and the mechanism of the inhibition has now been studied, since the compound is highly active and an understanding of such processes is of potential chemotherapeutic value.

Iodinin (I) is the di-*N*-oxide of a dihydroxyphenazine. The positions of the hydroxyl groups



I

are unknown but Clemo & McIlwain [1938] have suggested formulae in which co-ordination or tautomerism can occur, and have excluded the 2:3- and 2:5-compounds. The *N*-oxidic structure has been confirmed by resynthesis of iodinin from the dihydroxyphenazine produced by its degradation [McIlwain, 1943].

Natural antagonists of the anti-streptococcal action of iodinin

Characterization of materials or conditions which alter the action of antibacterial agents have been of value in indicating the extent and limitations of the possible practical use of these agents. In addition, such studies have suggested the modes of action of some synthetic bacterial inhibitors and this understanding has led to the preparation of other inhibitory compounds [McIlwain, 1942]. Some antibacterial agents induce new nutritional needs: in such cases media adequate for normal growth of organisms but rendered unsuitable by the inhibitor are made adequate again by addition of further materials. The action of such reversing materials was in some cases shown to be due to single compounds which were related to specific processes affected by the inhibitors. Iodinin, a natural antibacterial agent of a novel type, has now been subjected to such nutritional analysis. *Streptococcus haemolyticus* has been used as test organism as it was the most sensitive of those previously studied. In a medium which was as far as possible chemically defined, $0{\cdot}8$–$1{\cdot}2 \times 10^{-6} M$ iodinin was necessary to prevent growth of streptococci for a period of 6 days; in a peptone medium, and in one containing Marmite, such inhibition was produced by $1{\cdot}5$–$2 \times 10^{-6} M$ iodinin (Table 1). The relatively small difference between these minimal antibacterial levels permitted the use of the more complex media in subsequent work; this had the advantages of convenience and of making it more probable that the effects of reversing agents would be specific to the inhibitor (see p. 269).

In contrast to the inhibition caused by acriflavine or by the sulphonamides, inhibition by this pigment was relatively little affected by a variety of natural products, including microbial extracts, and plant and meat preparations (Table 2). The testing conditions employed little excess iodinin. This therapeutically valuable property may be due to limited distribution of materials related to the inhibitor, or to the type of inhibition which it produces [McIlwain, 1940*b*]. Blood (plasma or corpuscles),

Table 1. *Effect of medium on inhibition of streptococcal growth by iodinin*

Medium		Iodinin added	Growth after				
Type (see p. 269)	Addenda (mg./ml.)	$M \times 10^{-7}$	18–24 hr.	28–30 hr.	2 days	4 days	7 days
Amino-acid (*a*)		0	+ +	+ + + +	—	—	—
,,		3	+	+ + +	+ + + +	—	—
,,		6	0	+	+ + + +	—	—
,,		12 and more	0	0	0	0	0
Peptone (*b*) or marmite (*c*)		0	+ + + +	—	—	—	—
,,		3	+	+ + + +	—	—	—
,,		6	+	+ +	+ + + +	—	—
,,		12	0	0	0	+ + + +	—
,,		25 and more	0	0	0	0	0
(*b*)	+ serum, 100	100	+ + + +	—	—	—	—
,,	,, 20	100	0	+ +	+ + + +	—	—
,,	,, 100	10	+ + + +	—	—	—	—
,,	,, 20	10	+ +	+ + + +	—	—	—
,,	+ *rheum*, 2	100	+	+ + + +	—	—	—
,,	,, 0·4	100	0	+	+ + + +	—	—
,,	,, 2	10	+ + +	+ + + +	—	—	—
,,	,, 0·2	10	+	+ + + +	—	—	—

+, + +, etc., are proportional to the mass of growth, judged by visual comparison (see p. 269).

The most effective of the animal materials examined in cancelling the effect of iodinin, was required in quantities of about 0·2 g. to eliminate the activity of 5 μg. of iodinin.

The anti-streptococcal action of iodinin was not affected by addition of various metal salts with which higher concentrations of iodinin form lakes, nor by acid extracts of plant and animal ashes. Extracts of *Chr. iodinum* before pigmentation, and of other organisms insensitive to the inhibitor, did not antagonize its action.

Anthraquinones. The material first found to be active against iodinin in quantities small enough to be comparable with other drug-antagonists was *rheum*, which had been included in a survey of pharmaceutical materials. The activity of *rheum* was shared by other members of the groups of

anthraquinone purgatives (Table 2) which suggested that their anti-iodinin properties might be due to their therapeutically active constituents. Some of the active material in aqueous extracts of *rheum* was removed by extraction with ether from acid but not from alkaline solutions; from the acid-ether extracts it was removed by aqueous alkalies (Table 3). This treatment also separated hydroxy-

Table 2. *Anti-iodinin activity of natural materials*

Material	Quantity of substance (mg. or ml./ml. iodinin-containing medium (*b*)) needed for:— ($2 \times 10^{-6} M = 0{\cdot}5\,\mu$g./ml. iodinin had been added to medium (*b*))	
	Complete neutralization	Delayed or partial neutralization
Blood (human)	20, 20	4
Plasma (human)	50, 20	10, 5
Serum and plasma (horse)	100, 20	20, 4
Corpuscles	100	20
Meat (ox, hot extract)	(not by 100)	100, 50
Liver (ox, hot extract; and proprietary extract)	,,	100, 100
Urine (human, 3 specimens)	(not by 100, 20, 4)	
Potato, turnip (hot extracts)	,,	
Beer wort (3 specimens, used in growth of *Chr. iodinum* for pigment formation)	,,	
rheum (hot extract, referred to original weight)	2, 2	0·4, 0·08
Aloes (hot extract, referred to original weight)	0·5	0·1
Senna pods (hot extract, referred to original weight)	5	1, 0·4
Madder (hot extract, referred to original weight)	10	2
Yeast, boiling extract	(not by 100, 40)	40, 20
Yeast, hot extract [Warburg & Christian, 1938]	,,	40, 10
Yeast, plasmolysate (with acid ether)	,,	20
Yeast, autolysate (marmite)	,,	20, 10
Yeast, autolysate concentrates (5) active in antagonizing acriflavine inhibition [McIlwain, 1941*b*]	(not by 100, 20, 4)	
Escherichia coli, *Proteus vulgaris*, and *Chr. iodinum* culture filtrates and hot extracts	,,	

Table 3. *Properties of anti-iodinin materials in serum and* rheum*; activities of some concentrates and pure compounds*

Material	Quantity of substance (molarities or mg./ml. iodinin-containing mediums (*b*) and (*c*)) needed for:— ($2 \times 10^{-6} M = 0{\cdot}5\,\mu$g./ml. iodinin had been added to mediums (*b*) and (*c*))	
	Complete neutralization	Delayed or partial neutralization
rheum hot extract (weights refer to original *rheum*)	2	0·4
rheum acid-digest (weights refer to original *rheum*)	0·4	0·2
rheum ether extract of acid digest, and alkaline extracts of the ether (weights refer to original *rheum*)	2	0·4
rheum ether extract of acid digest, and alkaline extracts of the ether (weights refer to extractives)	0·03	0·006
Aloin	0·004	0·0008
Anthraquinone; anthraquinone-2:6-disulphonate	(not by $10^{-5} M$)	
1:2-Dihydroxyanthraquinone (alizarin)	(not by $2 \times 10^{-6} M$)	
1:4-Dihydroxyanthraquinone (quinizarin)	4 to $20 \times 10^{-7} M$	$4 \times 10^{-7} M$
1:5-Dihydroxyanthraquinone (anthrarufin)	$2 \times 10^{-5} M$	$4 \times 10^{-6} M$
1:8-Dihydroxyanthraquinone (chrysazin)	$2 \times 10^{-6} M$	$4 \times 10^{-7} M$
1:2:4-Trihydroxyanthraquinone (purpurin)	Not by $10^{-5} M$	$10^{-5} M$
1:2:7-Trihydroxyanthraquinone (anthrapurpurin)	(not by $10^{-5} M$)	
Serum (ox)	20	4
Serum after extraction with light petroleum	(not by 100, 20, 4)	
Serum petroleum ether extract, with and without residue	,,	
Vitamin K_1 concentrates	(not by 0·05)	0·05
2-Methyl-1:4-naphthaquinone	0·00032; $2 \times 10^{-6} M$	2·5 to $5 \times 10^{-7} M$

anthraquinones from the drug; the crude preparation so obtained was active in quantities of from 0·006 to 0·03 mg./ml. Aloin, which consists of the almost pure glucosides of aloe-emodin (1:8-dihydroxy-3-hydroxymethylanthraquinone), was even more active (4 to 0·8 μg. antagonizing 0·5 μg. of iodinin). Some commercially available hydroxyanthraquinones were examined, with results recorded in Table 3; at least three were highly active (1 to 0·1 μg. antagonizing 0·5 μg. of iodinin).

Serum and compounds of vitamin-K activity. Anthraquinone derivatives are not known to occur in normal blood, and the properties of the iodinin-antagonizing material from this source—which is of greatest importance in relation to the possible use of iodinin—were accordingly examined. The inhibitory activity of serum was lost on digesting with acid under conditions which hydrolysed anthraquinone glucosides. Extraction with petroleum ether destroyed the activity, but material active alone or in combination with the extracted serum could not be recovered from the petroleum extract. Recovery was attempted by washing the solid petroleum extractives with water, alkali, or ethanol and filtering or autoclaving the extracts in order to sterilize them. Such filtration did not affect the activity of whole serum. Alkali did not extract an active material from unevaporated petroleum ether extracts, nor was extraction of the active material from serum prevented by alkali. The iodinin-antagonists in serum are thus not hydroxyanthraquinones or other phenolic or acidic compounds, but are fat soluble. Such solubility may explain the apparently limited distribution of iodinin-antagonists in natural materials, as most of these have been examined as aqueous extracts because hitherto, almost without exception, bacterial nutrients have been found to be water-soluble.

One exception to the general rule of water solubility appeared relevant to the present investigations. Woolley & McCarter [1940] showed that K-vitamins and 2-methyl-1:4-naphthaquinone increased growth of Johne's bacillus. As compounds of vitamin-K activity exist in serum and bacteria [Almquist, Pentler & Mecchi, 1938] and bear some relation to the previously examined class of iodinin-antagonists, they were tested with respect to their power of neutralizing the inhibitory action of iodinin. Concentrates of vitamin K_1 (from grass, and of about 10 % purity) and K_2 (from putrified fish meal) were given by Mr F. A. Robinson of Glaxo Laboratories, to whom I am much indebted. Difficulty was experienced in obtaining them in forms suitable for bacteriological testing and their activity was variable; a filtered aqueous-alcoholic solution of the K_1 preparation, at a concentration corresponding to 0·1 mg./ml. of the specimen, was however active. The water-soluble 2-methyl-1:4-naphthaquinone possessing vitamin K activity was next tested, and its activity found to be comparable to, or greater than, those of the hydroxyanthraquinones previously examined (Table 3). Table 4 gives details of the inhibitory powers of different mixtures of iodinin and the quinone. Growth of streptococci in the presence of concentrations of iodinin greater than the minimally effective dose, was restored by roughly proportional increases in the concentration of the naphthaquinone which, when sufficiently high, annulled the effect of a saturated solution of iodinin. Solutions of 2-methylnaphthaquinone stronger than $10^{-4}M$ were sometimes themselves inhibitory of streptococcal growth.

Table 4. *Mutually neutralizing quantities of iodinin and 2-methyl-1:4-naphthaquinone.*

	Time (days) before appearance of visible growth in medium (*b*) containing: 2-methylnaphthaquinone (*M*)					
Iodinin (*M*)	4×10^{-6}	2×10^{-6}	1×10^{-6}	5×10^{-7}	$2{\cdot}5\times10^{-7}$	$1{\cdot}2\times10^{-7}$
2×10^{-5}	1½	2	>7	>7	—	—
1×10^{-5}	¾	2	3	>7	—	—
5×10^{-6}	—	1½	1¼, 2	3, 7	—	—
$2{\cdot}5\times10^{-6}$	—	¾	¾, 1½	1¼, 2	>7	—
$1{\cdot}2\times10^{-6}$	—	¾	¾	¾, 1¼	>7	>7
6×10^{-7}	—	—	—	¾	3	>7
3×10^{-7} and less	—	—	—	¾	¾	¾

The relatively small difference in the active concentrations of the more potent hydroxyanthraquinones and of 2-methyl-1:4-naphthaquinone, is in contrast to their activities as anti-haemorrhagic agents. Though structural requirements for vitamin-K activity are less exacting than those for most other types of vitamin activity, and several hydroxyanthraquinones are anti-haemorrhagic [Martin & Lischer, 1941] the amounts of the latter needed by chicks are 100 times greater than that of 2-methyl-1:4-naphthaquinone, and their activities are thought by the authors to be due possibly to their breakdown products.

EXPERIMENTAL

Iodinin. A specimen of the pigment was given us by Prof. G. R. Clemo and for later work it was prepared by growth of *Chr. iodinum* by the method of Dr J. G. Davis

[1939], to whom I am indebted for practical details and a culture of the organism. The pigment was strongly adsorbed during sterilizing filtration, and sterile solutions were prepared by autoclaving weighed quantities in plugged tubes and dissolving in N NaOH. The pigment was kept for no more than a week or two as a $M/100$ or $M/500$ solution in $M/5$ NaOH, and dilutions for testing were prepared from this. With solutions of $10^{-4}M$ or more, iodinin separated if the alkalinity fell below that of $M/5$ NaOH, and its Na salt tended to separate from the alkaline stock solutions.

Media. (*a*) The amino-acid medium was Medium III of McIlwain [1940*a*] containing thiolacetate and pantothenate concentrates.

(*b*) The peptone medium was that of McIlwain, Fildes, Gladstone & Knight [1939] based on glucose-peptone, with glutamine ($4 \times 10^{-4}M$) in place of the then unidentified factor. Oxoid peptone was the most dependable of those examined, and following the findings of p. 268 the solid peptone was extracted with light petroleum for 20 hr. in a Soxhlet apparatus before use.

(*c*) Before a suitable peptone was found, a medium was used consisting of (1) the amino-acids of Gladstone's [1939] medium; (2) mixture *A* of McIlwain *et al.* [1939]; (3) 0·25% of marmite, which had previously been extracted as a 10% solution, 6 times with its own volume of light petroleum, and (4) glutamine ($4 \times 10^{-4}M$), riboflavin ($10^{-7}M$), vitamin B_6 ($10^{-6}M$) and pantothenate ($10^{-7}M$).

Organisms and conditions of growth. The test organism was either the Richards strain of *Str. haemolyticus* or a group G organism; other strains behaved similarly. About 1000 organisms/10 ml. of medium were inoculated by suitable dilution of opacity-standardized suspensions from 1-day serum-agar slopes. A typical test comprised one tube of complete medium, others to which additions of different concentrations of iodinin had been made, and still others with further additions of varying quantities of materials to be examined for iodinin-antagonizing properties. The inocula were added last, together with other addenda common to all tubes; incubation was at 37° and normally in 5% CO_2-air. Records of such an experiment are given in Table 1 in which the + signs are proportional to the mass of growth, judged at intervals, by visual comparison. In common with other inhibitors iodinin caused morphological distortion in streptococci which grew in the presence of partially inhibiting concentrations. On subculture such organisms gave normal streptococci.

Natural antagonists

Blood was collected sterilely with citrate or oxalate as anticoagulant and the growth occurring in its presence followed microscopically. When serum was separated sterilely its anti-iodinin activity was the same as that of filtered specimens. Serum fractions were sterilized by filtration. Corpuscles were washed with saline and lysed before testing.

Anthraquinone purgatives. The crude extracts were prepared by twice heating the drugs at 100° with 20 parts of water. Two specimens of *rheum* were treated similarly; acid digestion was effected by refluxing 1 g. with $2N$ H_2SO_4 (20 ml.) in N_2 for 1 hr., specimens for test being diluted, neutralized and autoclaved. Ether extraction was carried out 6 times on the acid digest with an equal volume of solvent, of which part was evaporated and part re-extracted with N NaOH.

Quinones. All were commercial specimens; those found active were repeatedly crystallized from aqueous ethanol. Solutions of the less soluble ones were prepared by dissolving in a little ethanol, adding this to a large volume of hot water, and were sterilized by autoclaving. Vitamin K_1 concentrates (5 mg.) were dissolved in ethanol (5 ml.), water added to about 60% (by vol.) and the solution sterilized by filtration, and washed through the filter with a little ethanol.

DISCUSSION

Modes of action of iodinin and its antagonists

The antagonistic quinones. The manner in which the quinones act was considered in relation to four known types of *in vitro* relation between antibacterial agents and substances which antagonize them, viz. (1) reaction between inhibitor and antagonist in absence of the organism [cf. Oxford, 1942]; (2) interaction which takes place only in presence of the affected organism; (3) the anti-inhibitor action of substances which promote growth under the conditions of testing [McIlwain, 1941*b*; 1942] and (4) other cases, in which it must be concluded that the antagonist is more specifically related to the process of inhibition.

Iodinin antagonism is not by mechanism (3), as streptococcal growth was optimal during testing on media (*b*) and (*c*), and similar results were obtained in three distinct media; the varied inactive addenda of Table 2 also confirm this. With respect to (1) and (2), six batches of medium (*b*) of final vol. 50 ml. were prepared, all of which had a concentration of $10^{-5}M$ with respect to iodinin and 2-methylnaphthaquinone. Of these, two (*A*) were inoculated and incubated normally; two (*B*) were incubated with (*A*) but not inoculated, while two (*C*) were immediately extracted 3 times with 3 ml. $CHCl_3$. On the second day, (*A*) and (*B*) were similarly extracted, all the extracts were dried (Na_2SO_4) and were compared colorimetrically with standard iodinin solutions. The red iodinin colours of (*B*) were 95–100% of (*C*) and of the original iodinin but (*A*) was less than 10% of these. 2-Methylnaphthaquinone was also extracted but did not obviate these conclusions.

Interaction (1) can therefore be excluded but that postulated under (2) does occur. The disappearance of pigment is probably due to reduction, for although dihydroxyphenazine or other reduction products were not isolated, phenazine and $N.N'$-dihydrophenazine were separated from a similar experiment in which phenazine di-N-oxide [McIlwain, 1943] was used in place of iodinin. Dihydroxyphenazine and dihydrodihydroxyphenazine derived chemically from iodinin did not affect growth of streptococci when added to the test media in concentrations of 10^{-5} to $5 \times 10^{-4}M$. Reduction may therefore be the mode of inactivation of iodinin; it was sometimes found to be less active anaerobically. Iodinin

($10^{-5}M$) was also changed to yellow non-inhibitory material on incubating with thiolacetate ($M/100$; but not by $M/500$) or with ascorbic acid ($M/500$; not by $M/5000$), though these interactions are not comparable with such processes as cause inactivation of iodinin by 10^{-6} to $10^{-7}M$ solutions of quinones—not themselves reducing agents—which can take place only in the presence of bacteria. Pyocyanin ($10^{-5}M$; not $2 \times 10^{-6}M$) and methylene-blue ($5 \times 10^{-6}M$; not $10^{-6}M$), however, also antagonized iodinin.

The peculiarity of the antagonistic quinones then lies (*a*) in their greater potency than other redox systems, (*b*) in the probability that compounds with vitamin-K activity are responsible for the antagonistic action of serum, and (*c*) in the fact that the quinones are not known to function in a manner comparable with that of e.g. methylene blue or pyocyanin in other redox systems; conversely, the extracts examined for anti-iodinin activity contained many other natural carriers, but of these the quinones proved to be outstanding as regards iodinin-antagonism.

Streptococci and other organisms growing in the presence of sub-inhibitory concentrations of iodinin destroyed it. In such an experiment with streptococci and phenazine di-*N*-oxide, phenazine was again isolated. Such destruction is, therefore, a normal behaviour of growing streptococci; the special property of the antagonistic agent is to allow this growth in the presence of higher concentrations of iodinin.

Iodinin action. That such low concentrations of iodinin are anti-streptococcal makes it probable that it interferes with specific processes in the inhibited organisms. The more potent of its antagonists are structurally related to it, and their interaction with it and the organism may be similar to that suggested for other structurally related groups of inhibitory and antagonistic agents, e.g. for various amino-sulphonic and amino-carboxylic acids [McIlwain, 1942], for related amino-acids [Gladstone, 1939], and for indole acrylate [Fildes, 1941], naphthalene acrylate [Erlenmeyer & Bloch, 1942] and tryptophan. The case of iodinin is most like the first of these instances in that the shape and polarity of the antagonist molecule is very like that of the inhibitor, except that different atoms are substituted in important groups. If the suggestions which have been made in the above instances are adopted, a working hypothesis concerning iodinin action must assume the existence of 'sites', e.g. in essential enzymes, with which iodinin forms a type of combination involving *N*-oxidic groupings. Presumably it is essential to normal growth that these sites should be involved in the reaction of e.g. the essential enzyme with compounds similar to those quinones which are known to be antagonists of iodinin; but a sufficiently high concentration of iodinin must be able to displace the quinones and thus to inhibit growth.

The interaction between 2-methyl-1:4-naphthaquinone and iodinin is competitive within the limits of accuracy of Table 4, the quantity of iodinin necessary for complete inhibition of bacterial growth being 10 to 20 times that of the coincident naphthaquinone. Such an observation is consistent with the above hypothesis, but is not essential to it (cf. Clark, 1937). Though an observation such as this is explicable on the basis of other hypotheses, it is a phenomenon occurring in at least five of the instances quoted above. The hypothesis supposes that the quinones displace iodinin, thus allowing growth and bringing about destruction of the inhibitor; but alternatively streptococci with the aid of the quinones may change iodinin and so allow growth to be resumed. If iodinin can become attached to systems concerned with hydrogen transport in the organism, these alternatives, and those of (2) and (4) in A, above, approximate to one.

The structural relationship between iodinin and the antagonistic quinones has been used as a model in the preparation of quinoxaline di-*N*-oxides related to compounds of anti-haemorrhagic activity [McIlwain, 1943]. The di-*N*-oxides were in all cases found to be inhibitory at concentrations at which their parent quinoxalines were inactive, though these concentrations were greater than those required in the case of iodinin.

SUMMARY

1. Iodinin, the di-*N*-oxide of a dihydroxyphenazine, partly inhibited growth of streptococci in concentrations of $3 \times 10^{-7}M$ and completely inhibited it at 1·2 to $2 \times 10^{-6}M$. Phenazine di-*N*-oxide also displayed inhibitory action, but higher concentrations of it were necessary.

2. Extracts of a wide variety of plant, animal and microbial materials had relatively little influence on the inhibitory action of iodinin, but a few annulled its action and this was probably due to their containing anthraquinone and naphthaquinone derivatives. Pure hydroxyanthraquinones, and 2-methyl-1:4-naphthaquinone antagonized iodinin ($2 \times 10^{-6}M$) in concentrations of from 5×10^{-7} to $5 \times 10^{-6}M$.

3. When organisms overcame the inhibition due to iodinin and phenazine di-*N*-oxide, in the presence or absence of the quinones, the *N*-oxides were destroyed, probably by reduction. Some other hydrogen-transporting systems and reducing agents also caused destruction of iodinin and allowed growth of the organisms, but these agents were needed in higher concentrations than was necessary in the case of the antagonistic quinones.

4. As no interaction between the *N*-oxides and quinones occurred in the absence of the organisms, the quinones must have participated to some degree in the metabolism of the latter. The participation may be no more intimate than that of being concerned with hydrogen transport, but the structural similarity between the *N*-oxides and quinones suggests that the two classes of compounds may function at common sites in the organism, and that the action of iodinin may be to inhibit systems normally concerned with such quinones.

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Factors Influencing the Nucleoprotein Content of Fibroblasts Growing *in vitro*

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Since Carrel [1912] first showed that the growth of tissue cultures *in vitro* could be stimulated by means of extracts of embryonic tissue, and that cultures could be maintained apparently indefinitely in such extracts, much speculation has arisen as to the chemical nature of the constituents of embryo juice responsible for the stimulation of cellular proliferation. Baker & Carrel [1926] came to the conclusion that activity lay in the protein fraction, and obtained active materials by precipitation of these with ethanol or CO_2. Rejecting the theory that embryo juice contained a specific hormone for cell proliferation, they later suggested [Carrel & Baker, 1926] that growth-activating substances are not preformed in embryo juice but are continuously made from its protein, perhaps in the cells of the cultures themselves, as the result of enzyme action. In support of this suggestion they pointed out that proteoses and higher protein degradation products have very potent growth-promoting properties, an observation which was confirmed and extended by Willmer & Kendal [1932]. Needham [1931] also rejected the view that any one particular factor was responsible for the growth-promoting properties of embryo juice, which he regarded as 'a special collocation of the right nutrient substances probably protein break-down products', but later [Needham, 1942] he did not exclude the participation of 'some carrier substance of nucleotide type'. However, Fischer [1939; 1940] prepared a very active growth-promoting factor from beef embryos by isolating the nucleoproteins. The activity seemed to be located in the nucleoprotein fraction containing the ribonucleic acids, but repeated reprecipitation of the nucleoproteins resulted in loss of activity. In the active fractions the phosphorus content was higher than could be accounted for by the nucleic acid content. Some active fractions contained sulphur, probably derived from chondroitin or mucoitin sulphuric acid complexes precipitated with the nucleoproteins. The presence of ribonucleic acids in rapidly growing tissues is now well recognized [Caspersson & Schultz, 1939; 1940; Mitchell, 1940; Schultz, 1941], and the nucleoproteins of several different embryonic tissues have been shown to contain ribonucleic acids [Davidson & Waymouth, unpublished results].

By the term 'growth', applied to cells *in vitro*, as distinct from mere survival of the cells with or without cell migration and even cell division, is

meant cellular proliferation with the formation of new protoplasm. A number of criteria of growth have been suggested, e.g. the area of the culture [Ebeling, 1921], the mitotic index [Willmer & Kendal, 1932], the mass of the culture [Meier, 1931; Laser, 1933*b*; Wilson, Jackson & Brues, 1942], its metabolic activity [Meier, 1931; Laser, 1932; 1933*a*; Lipmann, 1932; 1933] or the nucleoprotein content [Willmer, 1942] as determined by chemical estimation of the nucleoprotein P (N.P.P.). The last has appeared to us to be most satisfactory although, as Willmer [1942] points out, it suffers from the defect that nucleic acid or nucleoprotein is a cytoplasmic constituent in some tissues [cf. Caspersson & Schultz, 1940]. Moreover, its chemical determination is based on the assumption that when a tissue has been extracted with fat solvents and with dilute acid, the residual P is all present as nucleic acid. Some may be present as phosphoprotein P [Plimmer & Scott, 1908], but the amounts of this in most tissues, including embryonic tissue, is very low [v. Euler & Schmidt, 1934], or negligible [Masing, 1911].

The work of Brues, Tracy & Cohn [1942] with radioactive P, suggests that the above assumption may not be fully justified for all tissues, but in agreement with Willmer we have found that the estimation of the 'nucleic acid phosphorus' provides a satisfactory index of growth in the case of cultures from the embryo chick heart.

MATERIALS AND METHODS

As reported in a preliminary communication [Davidson & Waymouth, 1942] we have employed the general technique described by Willmer [1942], involving the use of constricted roller tubes made of Pyrex glass.

General. All the tests have been carried out with fresh explants from the heart of the 9-day chick embryo. No experiments have been carried out on cultures maintained as a pure strain. This must be kept in mind when considering the results. Plasma was obtained from young cockerels. The Tyrode solution used had the composition described by Willmer & Kendal [1932], and was sterilized by filtration through a Berkefeld candle. Chick embryo extract was prepared by pulping 9-day chick embryos in sterile graduated centrifuge tubes with an equal volume of Tyrode solution, freezing, thawing, centrifuging, and pipetting off the supernatant fluid into sterile storage tubes. This concentrated extract (total N=40–60 mg. %) was suitably diluted before use.

Control experiments. As the composition of plasma is variable, direct comparisons of the effects of two substances on the tissue N.P.P. are made only where control and test series are grown in plasma from the same sample at the same time. For a similar reason, when embryo extract with and without some supplement is tested, comparisons are drawn only when a single sample of extract is used throughout. The effect of any lack of uniformity, due to inherent variations in potential growth capacity between different hearts, is reduced by using in each test pieces from a mixed sample from 6 to 8 hearts.

Preparation of explants. Each of the embryo hearts was cut up into 20–30 small and approximately uniform pieces according to the usual aseptic tissue culture technique [cf. Strangeways, 1924]. Six pieces of tissue were implanted in 0·2 ml. sterile plasma in each roller tube. The tubes were set aside until the plasma had clotted and 0·5 ml. Tyrode solution was then added. The tubes were stoppered with sterile rubber bungs, and mounted on a drum which rotated, by means of a small electric motor, in the incubator at 38° at a speed of 1 revolution/min. With each revolution the fluid washed over the growing cultures.

Six to eight of the tubes contained a plasma clot but no tissue, and served as plasma blanks. The remainder contained 6 pieces of tissue each. As the drum carried a total of 20 tubes, each test involved the use of some 70–80 pieces of tissue.

Technique of experiments. The tubes were rotated, with Tyrode solution alone as fluid phase, for 2 days to allow traces of growth-promoting substances present in the fresh tissue pieces to be used up, and to allow the cultures to be as uniform as possible before addition of test materials. The 'residual growth energy' is reported by Trowell & Willmer [1939] to be exhausted after this time. The tubes were then opened and the fluid phase (Tyrode + serum + metabolic products) sucked off. The test proper then began.

To each tube was added Tyrode, embryo extract, or test substance, or a combination of two or three of these, the total volume being 0·5 ml. As many duplicates as possible were set up. The tubes were re-stoppered and rotated for a further 3 hr. to allow the fluid to permeate the plasma clot. Some of the tubes were removed at this time (time = 0) to give figures for the initial value of N.P.P. The remainder were allowed to run for a further period of time, usually 2 days (time = *t*).

At 'time *t*' the tubes were removed. By means of a projectoscope, images of the cultures could be either outlined on paper and the areas subsequently determined with a planimeter, or recorded in silhouette on Ilford Reflex Document paper, although the curvature of the roller tubes made very accurate projection impossible.

Estimation of nucleic acid phosphorus. The method of Willmer [1942] was again followed. The roller tube became a test tube, and lipid P and acid-soluble P were extracted by ethanol-chloroform and 0·1 *N* HCl respectively, according to the procedure of Berenblum, Chain & Heatley [1939]. The tissue remained in the plasma clot throughout this process. It was then ashed in the roller tube with 0·15 ml. 70% perchloric acid, and P was determined by the ultra-micro-method of Berenblum & Chain [1938]. As originally described, the method is time consuming, since each estimation must be done individually, but we have devised a new type of mixing vessel (Fig. 1) which saves time by allowing

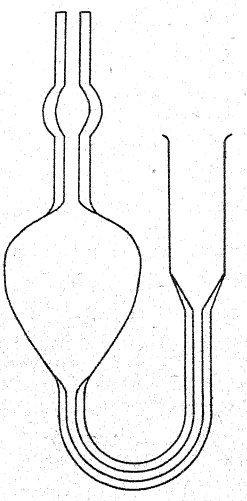

Fig. 1. Mixing vessel for phosphate estimation. ⅔ actual size.

a large number of estimations to be done simultaneously. A battery of seven (or more) of these vessels is set up in a rack so that at least six estimations and one standard can be carried through at the same time. The reagents are pipetted into the cup on the short limb and are transferred to the bulb by gentle suction applied to the long limb. After mixture of the aqueous and butanol layers in the bulb, the aqueous layer settles out and can be sucked off through a capillary pipette inserted into the bottom of the cup. The blue alcoholic layer is finally poured from the cup into a small tube graduated at 1 ml., and the colour is read off either in a colorimeter against a standard prepared simultaneously, or in the Hilger Spekker Absorbtiometer with micro-cups.

The figure for the N.P.P. in the tissue alone is determined by subtracting the appropriate plasma blank from the N.P.P. figure for each tube containing tissue. The index $(100Y)/X$ gives an indication of the increase in N.P.P. relative to the amount originally present where X and Y are the figures for the N.P.P. at 'time 0' and 'time t' respectively.

RESULTS

Tyrode solution alone as fluid phase

In agreement with Willmer [1942] we find a drop in N.P.P. in the course of the experiment (Table 1). In 23 different experiments the average figure for the initial N.P.P. was 1·28 μg./roller tube (after subtraction of appropriate plasma blanks). After 2 days the N.P.P. had fallen to 1·13 μg. (Table 1). No significant alteration in N.P.P. occurred in the tubes containing plasma and Tyrode without tissue.

Table 1. *Changes in N.P.P. (nucleoprotein P) in roller tubes containing Tyrode solution alone as fluid phase*

All tubes run for 2 days with Tyrode solution alone before test begins. Fresh Tyrode then introduced (time 0) and initial N.P.P. measurements made. Final N.P.P. estimation carried out 2 days later (time t).

	Initial N.P.P. at 'time 0' per roller tube μg.	Final N.P.P. at 'time t' per roller tube μg.	Diff.	No. of exps.
Tubes with plasma alone	0·29 ± 0·01	0·31 ± 0·02	+0·02	26
Tubes with tissue (corrected for plasma blanks)	1·28 ± 0·07	1·13 ± 0·06	−0·15	23

It is probable that this fall is due to the presence in the plasma of nucleases which break up the nucleic acid in such of the cells as die in the course of the 2 days. We have demonstrated the presence of such nucleases in cockerel plasma and serum. A mixture of 0·2 ml. cockerel plasma with 0·3 ml. veronal-acetate buffer (pH 7·6) and 0·1 ml. 2% thymonucleic acid or ribonucleic acid (as Na salts) was incubated at 37°. At suitable intervals portions were treated with an equal volume of 0·25% uranyl acetate in 2·5% $CCl_3.COOH$. The precipitate of unhydrolysed nucleic acid was centrifuged down, washed with 0·125% uranyl acetate in 1·25% $CCl_3.COOH$, and dissolved in Na_2CO_3 solution. Total P was determined in this solution by a modification of the method of Allen [1940]. Some spontaneous decomposition of nucleic acids takes place at pH 7·6, which was allowed for by a control series without plasma. Both types of nucleic acid are hydrolysed by plasma to compounds no longer precipitable by the uranyl acetate reagent (Table 2), but the ribonucleic acid suffers more decomposition than the thymonucleic acid. Cockerel plasma therefore contains enzymes of the nuclease type such as ribonuclease [Kunitz, 1940], and thymonucleodepolymerase [Fischer, Böttger & Lehmann-Echternacht, 1941; Greenstein & Jenrette, 1941], as well as nucleotidases, phosphatases, etc.

Table 2. *Influence of incubation with cockerel plasma on nucleic acids*

0·1 ml. 2% nucleic-acid solution (as Na salt) incubated with 0·2 ml. cockerel plasma and 0·3 ml. veronal-acetate buffer pH 7·6 at 37°.

	Hydrolysis (%)			
	Ribonucleic acid		Thymonucleic acid	
Time hr.	With plasma	Without plasma	With plasma	Without plasma
2	12·2	6·8	0	0
6	36·0	15·0	5·3	1·4
24	74·0	20·4	20·4	7·0

A similar fall in N.P.P. occurs during the first 2 days after the cultures are set up, during which period no measurements are normally made (Fig. 2).

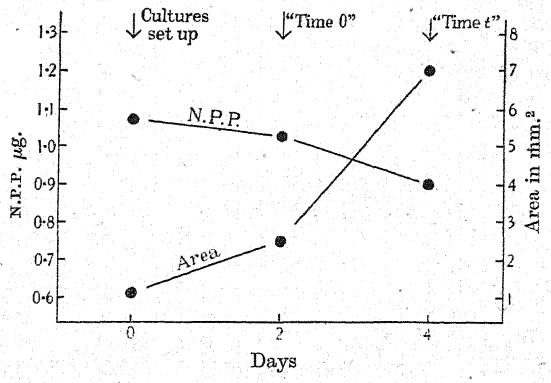

Fig. 2. Decrease in nucleoprotein P (N.P.P.) and increase in area in cultures grown in Tyrode solution alone for 4 days. N.P.P. in μg./roller tube (plasma blanks subtracted). Area in mm.²/individual culture.

Although the amount of nuclear material as measured by the N.P.P. diminishes continuously, the area of the cultures increases and may at the

Table 3. *Effect of various substances on nucleoprotein P (N.P.P.) content of tissue cultures*

All cultures grown for 2 days in Tyrode solution alone before introduction of test substance and estimation of initial N.P.P. (X). Final N.P.P. (Y) estimation 2 days later.

	Material under test	Fluid phase in roller tubes	Alteration in N.P.P. μg./tube	$(100Y)/X$
A	Tyrode solution	Tyrode solution alone	−0·15	88
B	Chick embryo extract	Embryo extract	+0·31	120
		Embryo extract after exposure to heat	+0·34	124
C	Crystalline ribonuclease	Tyrode solution alone	−0·14	87
		Crystalline ribonuclease in Tyrode solution (final conc. of enzyme = 9·4 mg. %)	−0·08	91
D	Crystalline ribonuclease	Chick embryo extract	+0·42	137
		Chick embryo extract + crystalline ribonuclease (final conc. = 9·4 mg. %)	+0·35	148
E	Crystalline ribonuclease (Dr Kunitz)	Tyrode solution alone	−0·13	76
		Chick embryo extract	+0·11	114
		Chick embryo extract + crystalline ribonuclease (final conc. = 12·5 mg. %)	+0·09	114
		Tyrode solution + crystalline ribonuclease (conc. as above)	−0·10	82
F	Crystalline ribonuclease (Dr Kunitz)	Tyrode solution alone	−0·07	92
		Chick embryo extract	+0·39	143
		Chick embryo extract + crystalline ribonuclease (final conc. = 50 mg. %)	+0·32	157
G	Crude ribonuclease in pancreatic extracts	Tyrode solution alone	−0·09	92
		Chick embryo extract	+0·45	136
		Chick embryo extract + preparation from pancreatin	+1·03	173
H	Mucinase	Chick embryo extract	+0·44	149
		Chick embryo extract + mucinase (final conc. of mucinase = 168 mg. %)	+0·07	107
J	Mucinase	Tyrode solution alone	−0·08	90
		Chick embryo extract	+0·37	143
		Chick embryo extract + mucinase (final conc. of mucinase = 160 mg. %)	+0·14	116
K	Embryo cartilage extract	Tyrode solution alone	+0·06	106
		Cartilage extract	+0·35	125
L	Anterior pituitary extract	Tyrode solution alone	−0·15	91
		Pituitary extract	−0·10	86

end of 4 days be as much as 6–10 times greater than it was when the cultures were first set up (Fig. 2) owing to the migration of cells from the original explant. The area of cultures from fresh explants is not, however, of great significance [Parker, 1938].

Chick embryo extract as fluid phase

(*a*) *The effect of normal extract.* When the Tyrode solution is replaced by embryo extract, during the second 2 days of the test (i.e. the days of the test proper), very marked growth of the tissue occurs, accompanied by a rise in N.P.P. (Table 3B). The final area, instead of being of the order of 7·0 sq. mm., is of the order of 20 sq. mm./culture.

The rise in N.P.P. is greater as the concentration of embryo extract increases. The effects of different concentrations of embryo extract are shown in Fig. 3. In this case some of the tubes were allowed to run for a further 2 days. During this time no further rise in N.P.P. occurred—in fact a slight fall was observed (Fig. 3). All tests have subsequently been restricted to 2 days after addition of the test substances.

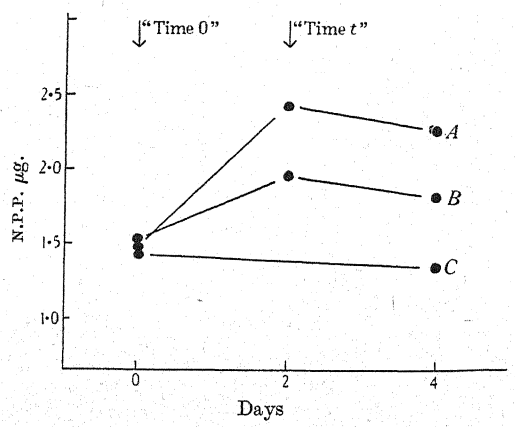

Fig. 3. Influence of different concentrations of embryo extract on the nucleoprotein P (N.P.P.) of tissue cultures. N.P.P. in μg./roller tube (plasma blanks subtracted). All cultures grown for 2 days in Tyrode solution alone before addition of embryo extract. Curve *A*: fluid phase in roller tube, embryo extract 30 mg. % N (approx.). Curve *B*: embryo extract 10 mg. % N (approx.). Curve *C*: fluid phase, Tyrode solution alone. The explants used in this experiment were rather larger than usual.

(*b*) *Effect of heat on embryo extract.* It is usually stated that the active principles of embryo extract are easily destroyed by heat [Carrel, 1913; Lasnitzki, 1937].

We have examined the effect of heating chick embryo extract to 100° for 10 min. To minimize the *p*H changes due to bicarbonate decomposition, the extract, prepared by pulping the embryonic tissue with water instead of Tyrode solution, was divided into two portions, one of which was heated while the other was kept as a control. The salt concentrations (including bicarbonate) in both extracts were then adjusted by adding appropriate amounts of sterile concentrated saline medium. Tissue cultures gained as much nucleoprotein P in this heated material as in the unheated control extract (Table 3B).

(*c*) *The effect of ribonuclease on embryo extract.* In view of Fischer's [1939] suggestion that the growth-promoting power of embryo extract is located in the ribonucleoprotein fraction, we have examined the action of the enzyme ribonuclease on the activity of chick embryo juice. Ribonuclease, which has been prepared in the crystalline state from pancreas by Kunitz [1940], attacks specifically the ribonucleic acids bringing about partial hydrolysis. The enzyme is strikingly thermostable and aqueous solutions can be sterilized by heat.

Chick embryo extract incubated overnight at 37° with crystalline ribonuclease (prepared from cattle pancreas) was tested on the tissue cultures, and showed no diminution in activity as compared with control embryo extract incubated without enzyme (Table 3 C, D). A sample of crystalline ribonuclease kindly supplied by Dr M. Kunitz gave the same result, even when the enzyme concentration in the roller tubes was as high as 50 mg./100 ml. (Table 3 E, F).

Ribonuclease in Tyrode solution alone appeared to have no effect on the living cells (Table 3 C, E). On the other hand, crude preparations of ribonuclease, prepared by extracting commercial pancreatin with 50 % acetone [cf. Dubos & Thompson, 1938], and sterilized by heating to 100° for 10 min., gave a very different response. Cultures grown in a mixture of this medium and embryo extract showed a characteristic dense type of growth with a sharply demarcated margin. Growth appeared to be abundant but cell migration was not so pronounced as in the controls grown in embryo extract alone, which had a considerably larger area. The cultures grown with the addition of the pancreatic extract showed a much greater increase in N.P.P. than did the controls (Table 3G). This increased growth is not caused by the ribonuclease in the pancreatic extracts, but is due to another factor, the nature of which is at present under investigation. It is destroyed by heating for 30 min. at 100° in feebly alkaline solution.

(*d*) *The effect of mucinase on embryo extract.* In view (1) of the large amount of mucin-like substances in embryonic material [cf. Baker & Carrel, 1926], (2) of the good growth-promoting power of embryonic cartilage extracts, and (3) of Fischer's [1940] suggestion that the active principle may contain S, we have tested the effect of a highly purified preparation of the enzyme mucinase [Madinaveitia, 1941] prepared from bull testicle, to see whether hydrolysis of some of the muco- or sulpho-polysaccharides in embryo extract affected its activity. We are grateful to Dr Madinaveitia for a sample of mucinase, which besides acting as a diffusing factor with hyaluronidase activity and with the power to reduce the viscosity of some mucopolysaccharides (of synovial fluid, vitreous humour, and umbilical cord, but not of saliva and gastric mucin), also had a low glucosaminidase activity. Testicular mucinase is stated by Meyer, Chaffee, Hobby & Dawson [1941] to hydrolyse chondroitin sulphuric acid, the mucopolysaccharides of skin, and the polysaccharide (or its H_2SO_4 ester) of the cornea as well as hyaluronic acid. The enzyme was dissolved in Tyrode solution and sterilized by passage through a small Berkefeld candle. A slight loss in N content resulted but the sterile solution still retained powerful enzyme activity as measured by the diffusion test in rabbits with haemoglobin [Madinaveitia, 1938].

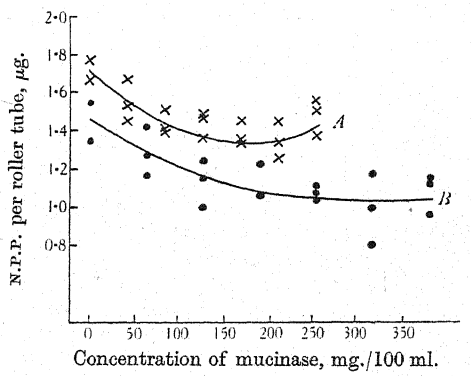

Fig. 4. The effect of increasing concentrations of mucinase on the nucleoprotein P (N.P.P.) of tissue cultures grown in embryo extract. N = 25 mg. % for embryo extract in all cases. Abscissa: N.P.P., μg./roller tube. Ordinate: final concentration of mucinase in roller tube, mg./100 ml. Embryo extract and mucinase incubated for 2½ hr. at 37° before being added to roller tubes. Curve *A* (test 74): cultures grown for 2 days. Curve *B* (test 76): cultures grown for 1 day. Plasma blanks not subtracted. All cultures grown for 2 days in Tyrode solution alone before addition of embryo extract and mucinase.

Embryo extract was mixed with the mucinase solution, and incubated at 37° for 2½ hr. before being added to the roller tubes. Embryo extract incubated at the same concentration without mu-

cinase served as a control. A slight precipitate sometimes appeared in the mucinase mixture.

The cultures grown in embryo extract plus mucinase showed a smaller increase in N.P.P. than did the controls (Table 3 H, J). As the effect seemed to vary considerably with the concentration of mucinase, experiments were carried out in which constant amounts of embryo extract were incubated with increasing concentrations of mucinase. The results are plotted in Fig. 4. It will be seen that the maximum effect was obtained when the mucinase concentrations were 170–250 mg. %. In one test (curve *A*) higher concentrations were rather less effective.

Extracts of cartilage from mammalian embryos

We have also employed extracts made from mammalian embryos, chiefly sheep embryos of 9–10 weeks, a stage at which growth is very rapid [Gurlt, 1847]. Cattle, pig and human embryos have also been used. As whole extracts prepared by mincing the complete embryos contained an undesirably large amount of blood, we have examined extracts from individual tissues. Embryo cartilage was found to give a very satisfactory extract.

The embryos were dissected and the cartilage of the vertebral column, skull, pelvic and shoulder girdles freed as far as possible from muscle and connective tissue. The cartilage was minced and the mince allowed to stand with an equal volume of water overnight at 0° before being filtered through muslin. The filtrate was centrifuged and treated with ½ vol. of double strength Tyrode solution. To check the salt concentration a chloride estimation was carried out and final small adjustments of salt concentration made as required.

Embryo cartilage extract could be sterilized by filtration through a Berkefeld candle, and kept quite well at 0° or, better, at −30°.

Results obtained with cartilage extract are shown in Table 3K.

The effect of pituitary extract

In view of the well-known action of extracts of the anterior pituitary gland in stimulating the growth of young animals [cf. Young, 1941] we examined the effect of pituitary extracts on the nucleoprotein content of fibroblasts *in vitro*. Saline extracts prepared according to the method described by Marks & Young [1940] were kindly supplied by Dr F. G. Young. The salt concentrations were adjusted to that of Tyrode before the extracts were tested. In no case did the pituitary extract cause any increase in the N.P.P. of the cultures (Table 3L).

This is in agreement with the findings of Trowell & Willmer [1939] who, contrary to Semura [1931], found that pituitary extracts had no growth-promoting power on tissue *in vitro*.

DISCUSSION

It must be emphasized that all the tests have been carried out in fresh explants from the 9-day embryo chick heart and not on pure strains of fibroblasts. The term 'fibroblast' is used here in the usual tissue-culture sense of cells characterized by a network arrangement [cf. Mayer, 1939].

Willmer's [1942] results were obtained mainly with chick periosteal fibroblasts, but in a few experiments with heart fibroblasts he found a fall in N.P.P. in Tyrode solution, while the effect of embryo extract was rather to prevent the fall than to cause a definite increase. In most of our experiments embryo extract produced a definite rise in N.P.P., and this effect was not appreciably diminished with extract which had been heated to 100° for a few minutes, or at 38° overnight. Although Carrel's [1913] original emphasis on the labile nature of the cative principles has been supported by later workers, e.g. Cracium [1931], Hueper, Allen, Russell, Woodward & Platt [1933], and Lasnitzki [1937] who found a decrease in activity on incubation at 70° for 10–30 min., recent workers have reported a greater heat stability, e.g. Paterson [1938]. Tennant, Liebow & Stern [1941] prepared in the ultracentrifuge a growth-promoting fraction from embryonic tissue the activity of which was not diminished by heating at 100° for several minutes. The effect of heated embryo extract on the N.P.P. of pure strains of fibroblasts is, of course, undetermined.

Ribonuclease attacks specifically ribonucleic acids, but its precise mode of action is unknown. Its action may be modified by the presence of protein to which the nucleic acid is bound. For example, Loring [1942] found that ribonuclease was unable to split up the nucleic acid of tobacco mosaic virus—a ribonucleoprotein—so long as the nucleic acid remained attached to the protein, although it could inactivate the virus by forming an enzyme-virus complex containing 14 % enzyme. This complex could subsequently be decomposed with liberation of intact and active virus. The claim of Schramm [1941] that intestinal nucleases were able to remove the nucleic acid from the virus leaving the protein intact was not confirmed by Cohen & Stanley [1942]. Although in our experiments ribonuclease even in high concentrations has not influenced the growth of cultures in embryo extract, the participation of ribonucleoproteins as growth-promoting agents cannot be completely excluded since the possibility of the reversible formation of enzyme-ribonucleoprotein complexes remains.

The nature of the factor present in a ribonuclease-containing extract of pancreatin, which causes such a marked increase in N.P.P., is not yet fully elucidated. The effect produced is not merely a quantitative one. Qualitative changes in the type of

growth produced are also found. There is some evidence that the factor may be a P-containing degradation product of protein.

The effect of mucinase is produced only when the enzyme concentration is very high. Both in this case and in the ribonuclease experiments, the enzymes remained active in the roller tubes and one cannot therefore exclude the possibility that, in the case of mucinase, the lower N.P.P. found in presence of the enzyme might be due to a direct action of the enzyme on the cells, rather than an action on the embryo extract. These experiments do, however, suggest that the muco- or sulpho-polysaccharides may be responsible for at least some part of the growth-promoting power of embryo extract.

SUMMARY

1. The effect of various substances on the nucleoprotein phosphorus content (N.P.P.) of fresh explants from the embryo chick heart growing *in vitro* in roller tubes has been examined.

2. Cultures grown in Tyrode solution alone show a fall in N.P.P. The addition of embryo extract to the cultures causes a definite rise in N.P.P., and this rise is unaltered when the embryo extract has been heated to 100° for 5 min.

3. Crystalline ribonuclease has no effect on the growth-promoting properties of embryo extract, but crude ribonuclease preparations from pancreatin contain a factor which causes a marked stimulation of nucleoprotein synthesis in the presence of embryo extract.

4. Cultures grown in embryo extract to which mucinase has been added in high concentration show a smaller rise in N.P.P. than control cultures grown in embryo extract without mucinase.

5. Extracts of mammalian embryos cause an increase in N.P.P. Sheep embryo cartilage extract is particularly effective.

6. Anterior pituitary extracts are without effect on the N.P.P. of the cultures.

We wish to express our gratitude to Mr E. N. Willmer of Cambridge for much valuable advice on tissue culture matters. A grant for scientific assistance from the Medical Research Council and an expenses grant from the Carnegie Trust for the Universities of Scotland to one of us (J. N. D.) are gratefully acknowledged.

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Microdiffusion Methods. Blood Glucose

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A manometric method for determining the true sugar in blood by measuring the CO_2 formed by yeast action was described by Holden [1937], and compared with the Somogyi [1927] modification of the method of Hiller, Linder & Van Slyke [1925] for determining the true sugar by a subtraction of the reducing material present after, from that present before, fermentation. A method for the estimation of blood glucose is here described in which the CO_2 evolved by yeast action on blood is measured by a microdiffusion technique. The whole procedure of fermentation, diffusion and titration is carried out in the one unit, the dimensions of which are smaller than those of the standard unit (it will be referred to as no. 2 size). The results are compared with those obtained by the Fujita & Iwatake [1931] modification of the Hagedorn-Jensen method on the same specimens of blood. In this modification the blood is deproteinized with Cd instead of Zn, and the method is claimed to give a reliable measure of the true blood glucose.

The microdiffusion conditions are different from the manometric, and an account is given of the influence of various factors before the method itself is described.

METHODS

1. *Microdiffusion apparatus*

Factors influencing CO_2 production and absorption. (*a*) *Dimensions of the microdiffusion unit.* A smaller unit than the usual standard type was considered suitable. The air volume of the standard unit—about 32 ml.—contains appreciable CO_2, and variations are likely to affect the results. Also minute leaks through defective lid contacts, which do not appreciably affect NH_3 determinations, are of importance when dealing with CO_2, and such are under better control in the smaller unit with its wider surface of contact. A smaller standard unit (no. 2) was therefore constructed with the dimensions given in Fig. 1.

The absorption rate of volatile substances such as CO_2, which are only slightly soluble (compared for example with NH_3), depends largely on the depth of the liquid diffusion layer, and is but little influenced by wide variations in the area of the absorbing or emitting surfaces. A comparison in Fig. 2 is made between the CO_2 absorption rate for the larger and smaller units (curves *C* and *D*). The carbonate was acidified in the outer chamber, the fluid depth being 0·147 cm. in both small and large units (0·8 and 2·3 ml. respectively). The rates of absorption are similar particularly for the longer times, though the surfaces differ considerably.

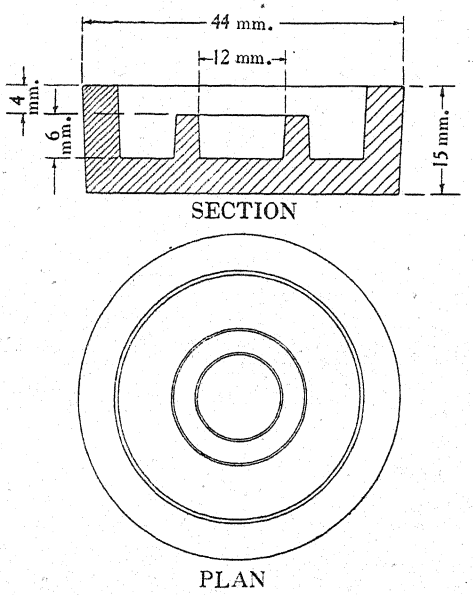

Fig. 1. Dimensions of the smaller (no. 2) microdiffusion unit (supplied by Messrs A. Gallenkamp, Finsbury Square, London, as Conway unit no. 2).

Curves *B* and *E* (Fig. 2) represent absorption curves with 0·4 and 1·6 ml. in the unit no. 2. Curve *A* is for 0·4 ml. with continual rotating of the unit (no. 2) and shows an absorption of 96 % in 5 min.

(*b*) *Autofermentation.* Besides the CO_2 coming from the fermentation of blood sugar, yeast steadily produces an appreciable quantity by autofermentation. Such autofermentation decreases with the age of the yeast suspension, the fall being most rapid in the first 24 hr. It is our custom when making a suspension to allow it to stand for 24 hr. at room temperature and then to place it in the refrigerator. Table 1 shows the influence of the age of the yeast suspension on the amount of

CO_2 produced by autofermentation from 0·5 ml. of a suspension (1 part in 3) of brewer's yeast (Guinness). Baker's yeast gave similar results, namely a marked decline in the autofermentation after 1 day at room temperature, and a steady fall

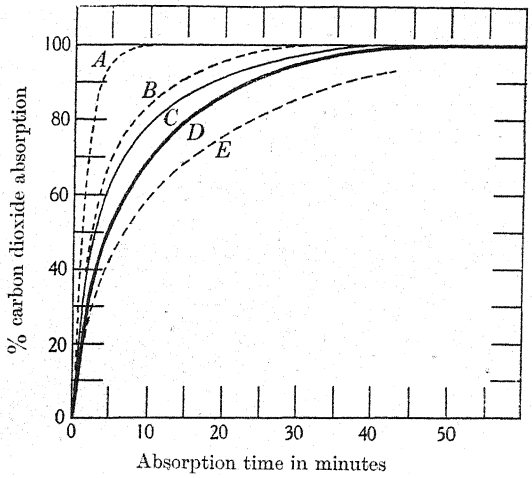

Fig. 2. Percentage absorption rate of CO_2 in the microdiffusion unit described in Fig. 1. The lines *A*, *B*, *D* and *E* are for absorptions with 0·4, 0·4, 0·8 and 1·6 ml. fluid volume in the outer chambers, respectively. For curve *A*, the unit was continually rotated. For all these curves, 0·2 ml. $Ba(OH)_2$ was contained in the inner chamber. Curve *C* shows the absorption rate for the standard unit (no. 1) with 2·3 ml. in the outer chamber (giving same depth as 0·8 ml. in the no. 2 unit) and 1·3 ml. $Ba(OH)_2$ in the central chamber.

subsequently. The autofermentation in the method described below is equivalent to 0·03–0·06 ml. of *N*/50 acid in 90 min., but it may be somewhat higher, depending on the yeast strain and the conditions in which the yeast was grown.

Table 1. *Change of autofermentation with age in a yeast suspension*

Conditions of CO_2 absorption	Age of suspension days	CO_2 formed (ml. *N*/50 acid)
0·5 ml. of 1 in 3 suspension of brewer's yeast in 2·5 ml. saline in outer chamber of standard unit (no. 1); absorption into $Ba(OH)_2$ in inner chamber for 90 min. at room temperature	0	0·75
	1	0·27
	2	0·18
	3	0·20
	4	0·09
	8	0·12

The rate of autofermentation is unaffected by wide changes in the external *p*H. Additions of HCl to yeast suspensions to give a concentration of 0·06*N* had no appreciable effect.

(*c*) *Yield of CO_2 from fermented glucose.* From the equation

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH,$$

1 mg. of glucose should yield 0·489 mg. CO_2 corresponding to 1·11 ml. *N*/50 acid. As is well known, less than this theoretical yield is obtained. We have found an average of 78 % with baker's yeast and of 92 % with brewer's top yeast (Guinness). Van Slyke & Hawkins [1929] found 71–79 % with baker's yeast, and Holden [1937] an average of 86 % with a certain type of brewer's yeast (L-Anheuser-Busch).

(*d*) *Effects of pH changes.* Over the range *p*H 3–5, produced by citric acid addition, there was no appreciable difference.

(*e*) *Effect of age and nature of fluid in which the stock yeast is suspended.* A stock baker's yeast suspension made up as 1 part in 10 of 0·5 % KH_2PO_4, left 1 day on the bench and subsequently stored in the refrigerator, showed little change in CO_2 production from glucose between 2 and 19 days. Thus, the recovery of CO_2 was 64 % of theoretical on the 2nd day and 73 % on the 19th day showing a slight rise with age; the fluctuation was around 70 % in the intervening days. After 27 days there was a marked reduction to 53 %. Other observations confirm the conclusion that yeast suspensions are serviceable for at least 2 weeks. No appreciable difference for about 2 weeks occurred if tap water or 0·9 % NaCl was substituted for 0·5 % KH_2PO_4 in suspending the yeast.

(*f*) *Variation in CO_2 yield from added glucose with different blood specimens.* The standard deviation from the mean value of a single formation of CO_2 from added glucose was 2·4 %, and 1·7 % for a duplicate. When blood samples from different human subjects were used instead of samples from the same subject, the variation of the CO_2 absorptions was not significantly different (2·6 %). This shows that varying the specimen of blood does not appreciably vary the CO_2 produced from a given amount of glucose. Wide variations in oxalate content of blood were also without effect. Cow's blood was found to give a result 5 % lower than that obtained with human blood.

(*g*) *Comparison of CO_2 formation in saline and in blood media.* We found that CO_2 formation from added glucose was a little higher when saline was substituted for the blood, and the variability greater. This could not be referred to *p*H effects. We have considered it advisable therefore to use the form of internal control as described below.

(*h*) *CO_2 formation in relation to quantity of glucose.* This was expected to show a linear relation up to a certain level. However, the CO_2 yield for small quantities of added glucose (1 mg./ml. blood) was proportionally less than for larger additions (2–4 mg./ml.). This effect was found to be suppressed by adding a little ethanol to the acidifying fluid introduced into the outer chamber. The total average CO_2 obtained in three sets of experiments in which

glucose (1–4 mg./ml.) was added to blood was 71 % of the theoretical. After 1 mg. addition it was 70 %, after 2 mg. 72 % and after 4 mg. 70 %, which values differ no more from the average than might be expected for the sampling error.

(*i*) *Acidifying mixture for use in the outer chamber.* With added ethanol the citric acid solution [Holden, 1937] produced a viscid mixture difficult to mix. Accordingly, we used dilute HCl, which produced a *p*H of 4–5 in the resulting satisfactory mixture.

(*j*) *Preformed CO_2 in blood and yeast.* The preformed CO_2 in the blood sample is rapidly removed on acidification and exposure to the air for 10 min. before yeast addition. The yeast suspension also contains preformed CO_2, and if many determinations are carried out there may be some difference in the CO_2 content of the yeast suspension added to the first and last samples. This possibility is overcome by adding 1·5 ml. of the yeast suspension to the inner chamber of one or more standard units (each of these 1·5 ml. additions is sufficient for seven samples) which are left exposed to the air for 15 min. before use. With the large surface exposed the preformed CO_2 quickly escapes leaving a small constant quantity dependent on the autofermentation rate.

(*k*) *Effect of glycolysis.* The present method, like Holden's, is independent of glycolysis up to 4 or 5 hr. exposure of blood samples to room temperature. If the samples are stored in the refrigerator much longer times may be allowed.

2. *Technique for determining fermentable sugar in blood*

(*a*) *Solutions required: N/20 $Ba(OH)_2$ with indicator.* Approximately *N*/20 $Ba(OH)_2$ containing 5 % of Universal indicator (B.D.H.). Before use some is run into a test-tube, and the 0·2 ml. pipette is dipped to the end of the tube. When half the solution has been used the tube is emptied and refilled with fresh solution.

N/50 HCl. For use in the horizontal burette.

Acidifying mixtures for outer chamber. (1) *N*/20 HCl containing 4 % ethanol. (2) *N*/20 HCl containing 4 % ethanol and 0·04 % glucose, which may be prepared by using a stock 2 % glucose in 2 % NaCl solution of which 2 ml./100 ml. are included.

Yeast suspension. Approximately 10 g. of yeast are made into a suspension with 100 ml. *tap* water, left for 1 day on the bench, and then placed in the refrigerator. It may be used after the 1st day for about 2 weeks. If the suspension is made in 0·5 % KH_2PO_4 it will be usable for a somewhat longer period.

Fixative for the lid of the diffusion apparatus. Good quality vaseline may be used, but a tragacanth fixative [Conway & O'Malley, 1942] has also been found very serviceable since it is water-soluble. 6 g. tragacanth are ground in a large mortar with the slow addition of 80 ml. of water; 30 ml. glycerol and 4 ml. *N* HCl are then added and mixed.

(*b*) *Procedure.* Into the inner chambers of some standard units (no. 1 size) 1·5 ml. of the yeast suspension are introduced. They are left on the bench while the others are being prepared. Duplicate samples of 0·1 ml. blood are then introduced into no. 2 units, 4 units being prepared for one specimen of blood. Two units are also set up with 0·1 ml. water. To all of these, except 2 of the 4 units with blood from the one specimen, 0·5 ml. of the acidifying mixture is added; this is mixed with the blood; 0·5 ml. of the acidifying mixture containing glucose is then introduced into the 2 remaining units.

Approximately 10 min. from the introduction of the acid, 0·2 ml. of the solution of $Ba(OH)_2$ plus indicator is introduced by a simple tube-pipette, and each unit is closed immediately by a lid smeared with tragacanth fixative. Into all the small units 0·2 ml. of the yeast suspension is now introduced, which is taken from the large units after stirring. This introduction is also made quickly from a 0·2 ml. tube-pipette, and after the unit has been closed the contents of the outer chamber are mixed by rotation. After 90 min. at room temperature the contents of the central chamber are titrated with *N*/50 HCl from the horizontal burette to *p*H 7·5–8·0.

(*c*) *Calculation.* The number of divisions on the burette corresponding to 1 mg. glucose/100 ml. blood is obtained by subtracting the mean titration for the 2 (of the 4) blood units which contain added glucose from the mean of the 2 without added glucose, this value being then divided by 200. For the remaining units the titration values are subtracted from the mean of those containing water, and divided by the result obtained above, giving the concentration as mg. glucose/100 ml. blood.

(*d*) *Example* (1) Mean titration of units with added glucose 11·0 (large divisions on burette)

(2) Mean titration of corresponding units without added glucose... 28·0

(3) Blank with water instead of blood ... 36·8

Concentration of glucose in the blood

$$=\frac{(36{\cdot}8-28{\cdot}0)}{(28{\cdot}0-11{\cdot}0)}\times 200=104 \text{ mg./100 ml.}$$

Other blood samples require the setting up only of duplicate units as in (2).

3. *Range of method*

The range of the method for the blood sample which includes the internal control is 300 mg./100 ml., but for other blood samples done at the same time it is 500 mg./100 ml.

RESULTS

Results obtained with the present method and with the Fujita & Iwatake [1931] modification of the Hagedorn-Jensen procedure on the same blood specimens are in good agreement. Those for the microdiffusion procedure are a little lower on the average, as might be expected, and the difference is more marked in the fasting subject. Table 2 gives a summary of the results. All determinations were made on oxalated blood, immediately after the specimen had been taken.

Table 2. *Comparison of blood sugar methods*

	Hagedorn-Jensen method (modification of Fujita & Iwatake) mg./100 ml.	Microdiffusion method mg./100 ml.
Non-fasting subjects		
1	103	108
2	137	128
3	110	112
4	154	157
5	99	95
6	107	94
Mean	118	118
Fasting subjects		
7	106	111
8	113	101
9	89	75
10	96	75
Mean	101	91
Weighted mean for the two groups	111	106

DISCUSSION

The present microdiffusion method involves less manipulation and fewer solutions than procedures such as the Hagedorn-Jensen, and is very suitable for a large number of determinations. Application of the method to a more complete investigation of the true glucose concentration of the blood, and to normal and pathological urines, etc., is still in an experimental stage, but the method for blood glucose appears sufficiently well developed for clinical use.

It should be noted that anti-glycolytic measures must not be used in collecting the blood, and that the method is independent of glycolysis up to 5 hr. at room temperature, or for one whole working day if the specimens are stored in the refrigerator.

SUMMARY

1. A microdiffusion method is described for the determination of blood glucose using 0·1 ml. samples. A smaller microdiffusion unit than the standard is used (Conway unit, no. 2).

2. The results obtained are very similar to those obtained with the Hagedorn-Jensen method modified by substitution of the Fujita & Iwatake [1931] procedure of deproteinization with cadmium instead of zinc, and which is claimed to give a measure of the true blood glucose.

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Effect of Caffeine and other Iminazole Compounds on Haematins and their Derivatives

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It is well known that haem combines reversibly with a great variety of nitrogenous substances [Anson & Mirsky, 1925], giving the corresponding haemochromogens. This reaction is recognized by the replacement of the reddish brown colour and diffuse absorption bands of haem by the red colour and very sharp bands of haemochromogen.

By the analysis of crystalline haemochromogens [Zeynek, 1910] and by spectroscopic titration [Hill, 1926] it was shown that haemochromogens are compounds of haem with two molecules of a nitrogenous substance. Haemochromogen is easily oxidized in air, forming, within a certain range of *p*H, the reddish brown compound of parahaematin.

Haemochromogen, like haem, combines with carbon monoxide forming a CO-haemochromogen in which one molecule of the nitrogenous substance is replaced by one of CO, according to the following equation:

$$\text{Porph. Fe}\begin{matrix}\text{N subst.}\\\text{N subst.}\end{matrix} + \text{CO} \underset{\text{light}}{\overset{\text{dark}}{\rightleftharpoons}} \text{Porph. Fe}\begin{matrix}\text{CO}\\\text{N subst.}\end{matrix} + 1\text{ N substance.}$$

This reversible reaction is light-sensitive, proceeding in the dark towards the right and in the light towards the left. Our knowledge as to the structure of haemochromogens can be summarized as follows. The four pyrrole nuclei of the porphyrin, united by four CH=groups, are arranged in a flat closed-ring system, the hollow space of which, in haematin, is occupied by the iron atom. Of the six covalencies of Fe four lie in the same plane and connect it with the nitrogens of the pyrrole rings, while one which lies above and the other below this plane connect it, in a haemochromogen, with the two molecules of a nitrogenous substance such as pyridine. One can see therefore that the Fe of haem will not be equally accessible to different nitrogenous substances which may react with free Fe.

Of the nitrogenous substances reacting with haem, the purine caffeine (1:3:7-trimethylxanthine) is of special interest. Purines or pyrimidines occur in nucleic acids, coenzymes (pyridine-adenine dinucleotide), the prosthetic group of enzymes (riboflavin adenine dinucleotide) and in adenylic acid. On the other hand, the iminazole ring, which occurs not only in purines but also in several other biological substances, especially in histidine, was recently suspected to be one of the links by means of which globin combines with haem to form haemoglobin. Finally, caffeine and some other purines and iminazole compounds have important pharmacological properties.

Although the reaction between caffeine and haem has twice been mentioned in the literature [D. Keilin, 1933; Barron, 1937], the nature of the compound thus formed was not properly understood nor were the reactions of other purines in this respect ever recorded.

The object of this investigation is to determine the nature of the reaction taking place between haem and other tetrapyrrolic compounds with caffeine and also with other purines and iminazole derivatives.

MATERIAL AND METHODS

Haemin used in this work was prepared from horse or ox blood by the Schalfejeff method. Caffeine was recrystallized from ethanol or from water. The reactions were followed spectroscopically, using for this purpose, according to requirements, one of the following four instruments:

(1) Microspectroscope ocular attached to a microscope which enables a rapid study of solutions in layers of different thickness examined in a test-tube, kept at an angle with the microscope stage. It also makes possible the study of mixtures in very narrow flat-bottomed tubes placed vertically above the condenser of the microscope. As these tubes require a very small amount of material, they are most suitable for the study of compounds available only in small quantities. Finally, by means of the comparative prism of the microspectroscope ocular, the absorption spectrum of one compound can be compared with that of another.

(2) Hartridge reversion spectroscope in a horizontal position, or attached to a microscope in place of an ocular, was used only for rapid determination of the exact position of absorption bands.

(3) Hilger Nutting spectrophotometer was used for more detailed study of absorption spectra in the visible region.

(4) Hilger quartz Spekker photometer was used for the study of the ultra-violet absorption spectra.

In all curves representing the absorption spectra the absorption coefficient ϵ is defined as $1/cd \ln I_0/I$, where c is the concentration of the pigment in g. mol./ml., d being the length of the tube in cm., I_0 and I the intensities of incident and transmitted light respectively.

RESULTS

Reaction of haems and derived compounds with caffeine

Reaction of caffeine and protohaem. A stock solution of alkaline protohaematin, prepared by dissolving about 12 mg. protohaemin in 50 ml. of 1% Na_2CO_3 or of 0·1 *N* NaOH, was diluted, according to requirements, either with alkali or with distilled water. $Na_2S_2O_4$ reduces the haematin to haem (two diffuse absorption bands). A little caffeine turns the reduced solution from reddish brown to a distinctly red colour, and its two diffuse bands are replaced by two much stronger bands: α, 570 mμ and β, 540 mμ. Both of these bands lie nearer the blue end of the spectrum than the corresponding bands of haem, and the intensity of the α-band is much greater than that of the β-band (Fig. 1). The γ-band is also intensified and shifts from 385 to 413 mμ. Moreover, the solution becomes very clear and does not precipitate on standing.

The minimum amount of caffeine required to complete this transformation was determined by adding known amounts of caffeine to standard solutions of haem, and comparing it spectroscopically with a solution of haem containing excess caffeine. It was thus found that the formation of the new compound requires at least 20 mol. caffeine/mol. haem.

Reaction of caffeine and haems other than protohaem. Caffeine reacts similarly with other haems such as mesohaem, haematohaem and deuterohaem. Caffeine reinforces and shifts the visible absorption bands of all these substances towards the blue end of the spectrum and prevents the precipitation of these pigments.

Reactions of caffeine and CO-protohaem. CO-protohaem is much more soluble than haem, giving a red solution with the absorption bands at 565 and 542 mμ, which has no tendency to precipitation. On the addition of caffeine to CO-protohaem the absorption bands of the latter become more intense and both bands are shifted considerably towards the red end of the spectrum (Fig. 1), their positions now being α, 590 mμ and β, 551 mμ. The solution has a tendency to become more cloudy and on standing the compound precipitates.

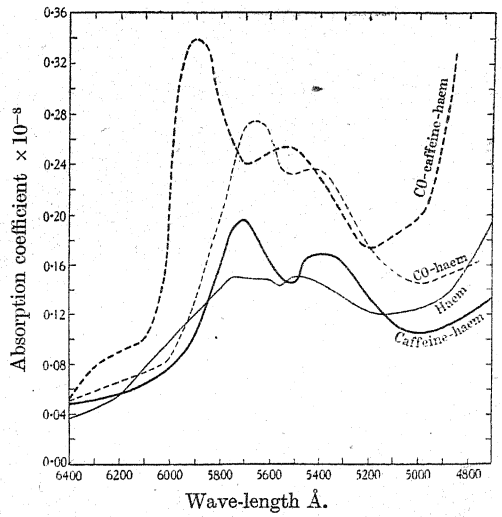


Fig. 1. Absorption spectra in the visible region of haem and CO-haem with and without caffeine.

The minimum amount of caffeine necessary for this reaction was determined in the usual way, and it was found that 1 ml. $1{\cdot}5 \times 10^{-3} M$ haem solution in 1 % Na_2CO_3 requires for the formation of this compound, CO and 0·1 ml. $1{\cdot}5 \times 10^{-2} M$ caffeine, while the addition of 0·1 ml. $0{\cdot}75 \times 10^{-2} M$ caffeine gives only about 30–40 % of this compound. This clearly shows that its formation requires 1 mol. of caffeine/mol. of CO-haem.

While a solution of protohaemin in 0·1 *N* NaOH, even when several days old, easily forms the caffeine CO-haem compound, the solution of haemin in 1–3 % Na_2CO_3 gives this compound only when freshly prepared. Such a solution after standing for 6 hr. or overnight forms mainly or solely a typical CO-haem, the absorption spectrum of which is not modified even on the addition of a large excess of caffeine. In the absence of CO, however, it forms with caffeine the characteristic caffeine-haem compound described above. Although the old solution of haematin in Na_2CO_3 gives typical haemochromogen and CO-haemochromogen compounds with different nitrogenous substances such as pyridine, nicotine, etc., yet the effect of caffeine and CO reveals that it differs markedly from the original fresh haematin solution.

That a protohaematin solution in Na_2CO_3 does undergo some important changes on standing is also shown by two other properties: it is no longer soluble in a mixture of ether and acetic acid and it does not give haemin crystals when treated with acetic acid and NaCl. On the other hand, if the haem solution is kept in a thoroughly evacuated Thunberg tube containing an alkaline solution of $Na_2S_2O_4$ in its hollow stopper it retains all the properties of a fresh solution of haem for several weeks. Such a solution, even after 5 weeks, gives the characteristic caffeine CO-haem compound with the α-band at about 590 mμ. Clearly the irreversible changes taking place in a carbonate solution of haematin are oxidative, and are irreversible even on addition of $Na_2S_2O_4$ for the purpose of reducing haematin to haem. The caffeine-CO-haem reaction therefore represents one of the most sensitive tests for the oxidative deterioration of a haematin solution, which can be detected even a few hours after its preparation.

Reaction of caffeine and CO compounds of other haems. We have seen that such compounds as haemato-, meso- and deuterohaem react with caffeine showing characteristic changes in their absorption spectra. These haems also react with CO, forming the corresponding CO-haems. On the other hand, the addition of caffeine to these CO-haems hardly affects their absorption spectra, a fact which shows that caffeine reacts solely with the CO-protohaem prepared from protohaemin crystals.

Reaction of caffeine and haemochromogens. Pyridine deuterohaemochromogen obtained by the addition of a minimum amount of pyridine to deuterohaem forms the best material for the study of this reaction. This haemochromogen shows two very strong absorption bands: α, 550 mμ and β, 520 mμ. On the addition of an excess of caffeine the bands at first become double and then the bands of pyridine haemochromogen are gradually replaced by two more diffuse but still strong bands of the caffeine-haem compound lying at α, 560 mμ and β, 530 mμ. On the addition of more pyridine these bands rapidly disappear, and are replaced by those of the ordinary pyridine haemochromogen. These reactions give the appearance of competition between pyridine and caffeine for the haem. Similar results can be obtained by treating with caffeine the haemochromogens obtained from other haems and other nitrogenous substances. The significance of this reaction will be discussed below.

Reactions between caffeine and porphyrins. Mesoporphyrin hydrochloride, dissolved in 0·1 % Na_2CO_3, gives a brownish red opalescent solution of alkaline porphyrin with a very characteristic diffuse absorp-

tion spectrum, the bands near the red end of the spectrum being the most distinct. On the addition of caffeine to this solution, the opalescence rapidly disappears, the solution becomes clear, its colour turns to salmon pink and the absorption spectrum now appears to be composed of sharp and distinct bands (Figs. 2, 3). This sharpening of the bands is accompanied by the displacement of the last three bands (II, III and IV) towards the blue end of the spectrum. About 20 mol. or more of caffeine are required per mol. of porphyrin to complete this change of colour and of absorption spectrum. The porphyrin thus modified remains in solution even on the addition of a little $CaCO_3$. Caffeine also affects other porphyrins such as proto- and haematoporphyrin in the same way, dispersing their colloidal solutions and changing their colour and their absorption spectra. Similar effects can, however, be obtained by treating porphyrins with solvents like pyridine and ethanol (Fig. 2), or with surface active substances such as sodium desoxycholate, sodium taurocholate, digitonin and sodium dodecyl sulphate which dissolve or disperse the aggregated molecules of porphyrin.

The reaction of porphyrins with caffeine shows a great similarity to the reaction of porphyrin with globin. Hill & Holden [1926] found that when haemato- or mesoporphyrin in dilute $NaHCO_3$ is added to a solution of native globin, the colour of the porphyrin changes from orange to pink and its absorption bands, previously diffuse, become very sharp and intense. Moreover, the solution thus obtained is no longer precipitated by $CaCO_3$. Hill & Holden considered that this reaction indicated the formation of a compound between porphyrin and globin, which suggests that 'the porphyrin residue plays a part in the formation of haemoglobin independently of the iron'.

This view was not, however, accepted by Haurowitz & Waelsch [1929], who found that although serum proteins affect the colour and the absorption bands of porphyrin in the same way as does globin, yet these proteins form no haemoglobin with haem. It is, on the other hand, possible that for the formation of haemoglobin the protein must combine both with the porphyrin and with the iron of haem, and that 'serum proteins' fail to give haemoglobin because in their native state they do not react with the iron of haem.

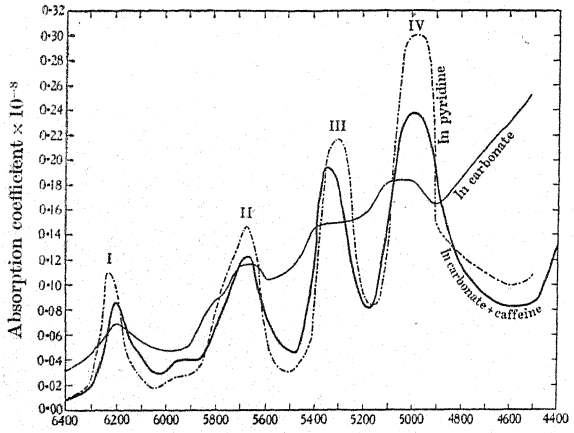


Fig. 2.

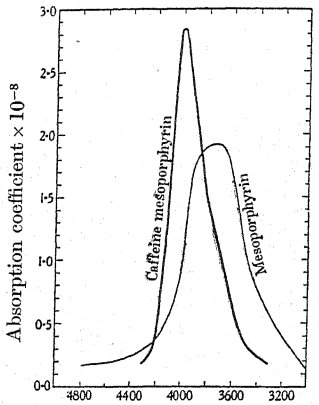


Fig. 3.

Fig. 2. Absorption spectra in the visible region of mesoporphyrin in weak carbonate solution with and without caffeine and in pyridine.

Fig. 3. Absorption spectra in ultra-violet region of mesoporphyrin in weak carbonate solution with and without caffeine.

Reactions of caffeine with metalloporphyrins. The best material for the study of this reaction is turacin. This pigment, described for the first time by Church [1870; 1892] as a copper porphyrin compound, was considered by Fischer & Hilger [1924] to be composed of copper and uroporphyrin I. Its structure was recently reinvestigated by Rimington [1939] who identified it as a compound of copper with uroporphyrin III, the natural isomer allied to the porphyrin of haemoglobin.

Turacin is very soluble in weak alkalis giving a clear purplish red solution with two absorption bands: α, 562·5 mμ and β, 526·0 mμ. On acidifying this solution, turacin changes its colour from purplish red to orange, and the two absorption bands are shifted towards the red end of the spectrum: α, 583 mμ and β, 542 mμ. The pigment gradually

aggregates until it forms a flocculent orange precipitate. This change in colour and absorption spectrum takes place even if the precipitation of turacin is prevented by the addition of a colloidal protector such as 0·2–0·4 % gum arabic. The absorption spectrum of turacin in feathers is the same as that of its acid suspension. It was clearly demonstrated [D. Keilin, 1926] that the two distinct absorption spectra of turacin are due to different degrees of dispersion of the pigment, the bands shifting towards the blue end of the spectrum as the degree of dispersion increases and towards the red end of the spectrum when the molecules of the pigment begin to aggregate. In fact, the absorption spectrum of turacin in acid ethanol and in aqueous alkaline solution is the same.

The dispersing properties of caffeine can therefore easily be tested with turacin. For this purpose a red feather of a Turaco bird is washed in ether and immersed in a very weak solution of ammonia, giving a characteristic purplish red turacin solution. On acidifying this solution with acetate buffer *p*H 4·5 the solution turns rapidly orange and the absorption bands are shifted towards the red end of the spectrum (α, 583 mμ; β, 542 mμ). If a small amount of caffeine is now added, the colour of the solution becomes purplish red and the absorption bands return to their initial position (α, 562 mμ; β, 526 mμ), although the solution remains distinctly acid. This clearly shows that caffeine disperses the aggregates of acid turacin probably by forming a complex with its porphyrin. Even an alkaline solution of turacin is affected by caffeine; this is shown by a perceptible intensification and a slight shift of its absorption bands further towards the blue end of the spectrum. Caffeine also intensifies and shifts the absorption bands of other metalloporphyrins such as manganese mesoporphyrin. It is interesting to note that although a caffeine-like effect on turacin can be obtained with solvents like ethanol and pyridine, it cannot be imitated by surface active substances or detergents such as bile salts or Na dodecyl sulphate.

Nature of reaction between caffeine and haem

We have seen above that the addition of caffeine to haem changes its colour from reddish brown to red and markedly affects its absorption spectrum by reinforcing and shifting its bands.

It is generally believed that the changes taking place in haem on the addition of a nitrogenous substance are always due to the formation of a haemochromogen compound, in other words to a direct combination of the Fe$^{\cdot\cdot}$ of haem with two molecules of this nitrogenous substance. In fact, the reaction between caffeine and haem was previously ascribed to the formation of such a caffeine-haemochromogen compound [D. Keilin, 1933; Barron, 1937]. A more detailed study of this reaction, however, does not support this view. On the contrary, it shows that caffeine reacts not with the iron but with the porphyrin of the haem, and caffeine-haem is therefore a more appropriate name for such a compound. This supposition is supported by the following considerations:

(1) Caffeine, unlike the haemochromogen-forming nitrogenous substances, even within the characteristic very limited range of *p*H, never forms a parahaematin compound with haematin.

(2) It is well known that haematin does not form a true solution even in alkali. It is always present in the form of molecular aggregates reaching a molecular weight of about 50,000 [Zeile & Gnant, 1940]. After reduction the aggregates of haem rapidly increase in size, and eventually form a precipitate. The diffuse absorption spectrum of haem is probably due to the formation of these large molecular aggregates. The clarification of the haem solution, and the reinforcement and shift of its absorption bands towards the blue end of the spectrum, caused by the addition of caffeine, can only be ascribed to the dispersion of these large molecular aggregates of haem. In fact, similar changes in haem can be observed on the addition of solvents such as ethanol, or of the previously mentioned surface active substances.

(3) That the dispersion of haem is probably due to complex formation between caffeine and the porphyrin of haem is strongly supported by the fact that caffeine reacts in a similar way with other metalloporphyrins such as turacin, as well as with free porphyrins.

(4) The effect of caffeine on haemochromogens suggests at first sight the possibility of competition between caffeine and pyridine for the Fe$^{\cdot\cdot}$ of haem. This competition is, however, an apparent one, as similar results can be obtained by using a suitable solvent or surface active substance.

Nature of reaction between caffeine and CO-haem

This remarkable effect of caffeine can be tentatively explained in two different ways:

(1) It is possible that caffeine combines directly with the Fe of CO-haem and forms CO-caffeine haemochromogen, although, as we have seen, it may not form with haem a caffeine haemochromogen. This supposition is supported by the following considerations:

(*a*) CO-haem is known to have a much greater affinity than the free haem for nitrogenous substances.

(*b*) Only one molecule of caffeine per molecule of CO-haem is required for the complete change in the absorption spectrum of the CO-haem. It is known, on the other hand, that the formation of CO-haemochromogen requires only one molecule of a nitrogenous substance per Fe atom of haem.

(*c*) The addition of caffeine to CO-protohaem shifts its absorption bands towards the red end of the spectrum and makes the compound less soluble. This is very unlike its effect on haem, on the metalloporphyrins and on porphyrins which, on the contrary, are dispersed and made more soluble. It is known, however, that while CO-haem is one of the most soluble derivatives of haematin in dilute alkali, CO-haemochromogen is a much less soluble compound.

(2) An alternative and more plausible explanation is that caffeine, as in the case of other tetrapyrrolic compounds, reacts with the porphyrin and not with the Fe of CO-haem. This is supported by the following considerations:

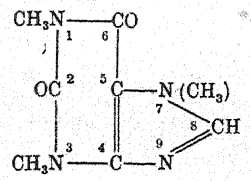
(*a*) The characteristic effect of caffeine on CO-haem is obtained only if the haem is prepared from a solution of protohaematin; it has no marked effect on the CO-compounds of other haems such as meso-, haemato-, or deuterohaem.

(*b*) Unlike CO-haemochromogen, the caffeine CO-haem compound is not affected by light.

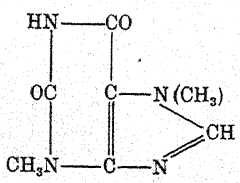
(*c*) On the addition of a solvent like ethanol, or of surface active substances such as bile salts, the characteristic absorption bands of caffeine-CO-haem are rapidly replaced by those of the ordinary CO-haem compound. Moreover, on the addition of pyridine, an ordinary CO-pyridine haemochromogen is formed.

Reaction of purines other than caffeine with tetrapyrrolic compounds

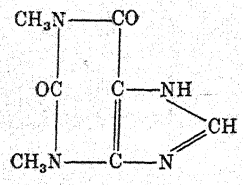
This study revealed the remarkable fact that caffeine and chlorocaffeine are the only purines which react with haem and its derivatives in



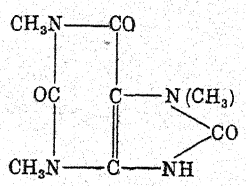
caffeine



theobromine



theophylline



1:3:7-trimethyl uric acid

the manner described above. The other purines examined in this respect were completely inert and negative results have been obtained with adenine, guanine, xanthine, hypoxanthine, paraxanthine, heteroxanthine, theophylline, theobromine, uric acid and at least twelve of its derivatives including 1:3:7-trimethyl uric acid. Yet the structures of three of these compounds, theophylline, theobromine and 1:3:7-trimethyl uric acid, closely resemble that of caffeine.

It seems that in order to react with haem or with a porphyrin, the purine must have the nitrogen atoms 1, 3 and 7 methylated; the hydrogen in position 8 may be replaced by chlorine but not by oxygen.

Effects of pyrimidine and iminazole compounds on haem and its derivatives

The three pyrimidines—thymine, cytosine and uracil—tested in the usual way, produced no effect either on different haems or on their porphyrins.

Iminazole compounds, on the other hand, are known to react with haematins and their derivatives. Küster & Koppenhöfer [1927] were the first to suggest that the iminazole ring of histidine probably represents the group by means of which the haem is attached to globin to form haemoglobin. The great affinity of 4(5)-methyl iminazole for haem with formation of haemochromogen, and for haematin with formation of parahaematin was shown by Holden & Freeman [1929]. They find it 'difficult to believe that the iminazole ring of histidine is the sole or even the chief group in proteins which combines with reduced haematin. It may well be, especially in the case of denatured globin, that some of the molecules of reduced haematin combine with two different types of nitrogen atom, one of which is in the iminazole ring'. Langenbeck [1932] obtained crystalline parahaematins of glyoxaline and 4(5)-methyl iminazole. The facts, (i) that certain iminazole compounds have such a great affinity for haematin, and (ii) that globin is so rich in histidine, suggest, according to Langenbeck, that histidine must play an important part in linking haematin to globin. Haurowitz [1935], however, pointed out that not all iminazole compounds have high affinity for haematin; histidine itself does not form a stable parahaematin compound nor on reduction does it give a haemochromogen; globin of invertebrate haemoglobins is not rich in histidine.

The comparative study of other iminazole compounds in respect of their reactions with alkaline porphyrin, haematin, haem, CO-haem and acid turacin were carried out on other purines (*v. sup.*), iminazole, iminazole 4:5-dicarboxylic acid, 1-methyl iminazole, benziminazole, 1-methyl benziminazole, histidine, histamine, ergothioneine, anserine, carnosine and pilocarpine. The results of these experi-

ments, which are shown in Table 1, can be summarized as follows:

(1) Caffeine and chlorocaffeine are the only purines which give the characteristic reactions described above. Other purines, including theobromine, theophylline and 1:3:7-trimethyl uric acid, do not react with either haematin, haem, other metalloporphyrins, or porphyrins.

histidine and β-alanine, forms with haem a typical haemochromogen but fails to react with porphyrins.

(5) The fact that globin [Hill & Holden, 1926] and serum proteins [Haurowitz & Waelsch, 1929] react with porphyrins in the same way as caffeine or pilocarpine, suggests that some of the constituents of these proteins must have certain properties in common with the 1-methyl iminazole derivative.

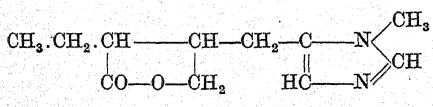
Table 1. *Reaction of different iminazole compounds with tetrapyrrolic compounds*

0 means no appreciable reaction observed.
'Caffeine effect' means dispersion, solution, reinforcement and shift of bands.

	Haematin	Haem	CO-haem	Alkaline porphyrin	Acid turacin
Caffeine and chlorocaffeine	0	Caffeine-haem	Caffeine-CO haem	Caffeine effect	Caffeine effect
Other purines	0	0	0	0	0
Iminazole	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
1-Methyl iminazole	Parahaematin	Haemochromogen	CO-haemochromogen	Caffeine effect	Caffeine effect
Iminazole 4:5-carboxylic acid	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Benziminazole	0	0	0	0	0
Methyl benziminazole	0	Haemochromogen	CO-haemochromogen	0	0
Histidine	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Histamine	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Ergothioneine	0	0	0	0	0
Anserine	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Carnosine	0	Haemochromogen	CO-haemochromogen	0	0
Pilocarpine	Parahaematin	Haemochromogen	CO-haemochromogen	Caffeine effect	Caffeine effect

(2) The other iminazole compounds, unlike caffeine, combine with the Fe of haematin, of haem and of CO-haem, forming respectively parahaematin, haemochromogen and CO-haemochromogen. Histidine forms these compounds with difficulty, while ergothioneine does not seem to form them at all.

(3) Of all the iminazole compounds tested, only pilocarpine, and to a lesser degree, 1-methyl iminazole (in addition to their ability to form parahaematins, haemochromogens and CO-haemochromogens), share with caffeine the property of reacting with and dispersing the porphyrins and metalloporphyrin compounds. In the alkaloid pilocarpine, the 1-methyl iminazole ring is attached solely by its carbon 5 to another ring which can be easily separated as homopilopic acid:



Pilocarpine

(4) The fact that with porphyrin pilocarpine reacts like caffeine and with haem like 1-methyl iminazole or even pyridine, shows that the failure of caffeine and of other purines to react with the $Fe^{\cdot\cdot}$ of haem and to give a haemochromogen compound, is probably due to the steric hindrance caused by the pyrimidine ring. It is also interesting to note that anserine, a dipeptide of methylated

However, the only iminazole compound so far known to occur in proteins is histidine which, as we have seen, does not react with porphyrins. Negative results in this respect were also obtained with other constituents of proteins such as alanine, arginine, aspartic acid, cystine, cysteine, glutamic acid, glycine, hydroxyproline, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine.

The observation that some proteins have a very strong 'caffeine effect' on aqueous solution of alkaline porphyrin, while no known constituent of these proteins shows such an effect, can be explained in two ways:

(1) The protein may contain an additional unknown constituent with the properties of the iminazole ring of caffeine or pilocarpine.

(2) The iminazole ring of histidine, when this amino-acid forms part of a polypeptide, may differ in certain properties from that of the free amino-acid, and may resemble more the iminazole ring of caffeine and pilocarpine.

More work, however, is required before one or other of these explanations can be definitely accepted and important information as to the chemical structure of proteins and the properties of polypeptide chains may be obtained by making use in these investigations of the reactions with haematins and their derivatives.

Physico-chemical properties of caffeine and its pharmacological action

It is well known that caffeine acts on the central nervous system, the kidneys, muscles and heart. The most important pharmacological action is that on urinary excretion, but there is no general agreement as to the mechanism of caffeine diuresis: various interpretations were critically reviewed by Verney & Winton [1930] and by Smith [1937]. Ellinger, Heymann & Klein [1921] and Brühl [1929] alone have made an attempt to correlate caffeine diuresis with certain physico-chemical properties of this drug, and only the results obtained by these workers will therefore be discussed. Ellinger *et al.* found that the rate of ultrafiltration of Ringer solution, mixed with serum, through a Bechhold collodion membrane (impermeable to proteins) is increased 60 % by the addition of as little as 1 in 56,000 caffeine. This is ascribed by these workers to the direct effect of caffeine on the properties of proteins, lowering their affinity for water, and not to the increased permeability of the filter membrane or glomeruli. This physico-chemical mechanism explains, according to Ellinger *et al.*, all the facts of caffeine diuresis. On the other hand, Brühl [1929] found that caffeine protects protein from precipitation by low concentrations of ethanol and increases the solubility of congo-red, and of certain amino-acids. Brühl ascribes these effects to the ability of caffeine to combine with proteins and other organic substances, and this seems to agree with some of the results obtained by other workers [Schüller, 1929; Labes & Rubenbeck, 1933]. He found also that the permeability to buffer solutions of collodion membranes impregnated with albumin or globulin, is much increased by the addition of caffeine. According to Brühl the diuretic effect of this drug is due to an increased permeability of glomeruli, a view previously expressed by Cushny & Lambie [1921]. Neither Ellinger *et al.* nor Brühl has compared the physico-chemical properties of caffeine which were observed with those of theobromine and theophylline. There is little doubt that the reactivity of caffeine and its solvent and dispersing properties discussed above are intimately connected with the physico-chemical properties of this drug which are considered by Ellinger *et al.* and by Brühl to explain the mechanism of caffeine diuresis. We have seen, on the other hand, that in its physico-chemical properties as revealed by reactions with tetrapyrrolic compounds, caffeine differs very markedly from theobromine and theophylline. If therefore these properties of caffeine have a direct bearing on its physiological action, the diuresis induced by the three purine derivatives cannot be ascribed to one and the same mechanism. All this shows that the intimate mechanism of caffeine diuresis will probably only be solved by a comparative physico-chemical and physiological study of the three diuretic purines carried out in the same way and under the same conditions.

SUMMARY

1. On the addition of caffeine to an alkaline solution of protohaem, the solution turns from reddish brown to red, and its two diffuse absorption bands are replaced by much stronger bands lying nearer the blue end of the spectrum. The solution loses its opalescence and on standing does not precipitate. A similar effect is produced by caffeine on other haem compounds such as meso-, deutero- and haematohaem.

2. Contrary to the view held by previous workers, the compound thus obtained is not a caffeine-haemochromogen. In this reaction caffeine combines, not with the $Fe^{\cdot\cdot}$ of haem, but with its porphyrin as the following evidence shows: (*a*) Addition of caffeine to an aqueous alkaline solution of porphyrin changes its colour from orange to salmon pink, and greatly intensifies and shifts its absorption bands. The solution loses its opalescence and does not precipitate on standing. (*b*) Caffeine intensifies and shifts the absorption bands of metalloporphyrin compounds. It disperses and dissolves a suspension of acid turacin, the colour and the absorption spectrum of which become similar to those of an alkaline solution of this pigment, although the reaction of the solution remains acid. (*c*) About 20 mol. of caffeine per mol. of haem or porphyrin are required to produce the complete transformation. (*d*) The effect of caffeine on haems and on porphyrins can be obtained by treating these pigments with solvents, or with surface active substances such as bile salts or sodium dodecyl sulphate.

3. Caffeine reacts with CO-protohaem shifting its two absorption bands from α, 565 mμ and β, 542 mμ to 590 mμ and 551 mμ respectively. Only one molecule of caffeine per molecule of CO-protohaem is required to complete this change.

4. This effect of caffeine can be obtained with CO-protohaem prepared from a fresh solution of haematin. After standing in air for 24 hr. or less, the solution in carbonate fails to give the characteristic caffeine CO-haem compound, while the same solution, if kept anaerobically, gives this reaction even after several weeks. In carbonate solution haematin is apparently very unstable in air.

5. Caffeine gives no reaction of this nature with CO-haem compounds obtained from other haematins such as haemato-, deutero- and mesohaematin.

6. Two possible explanations as to the mechanism of the reaction between caffeine and CO-haem are proposed and discussed.

7. Caffeine and chlorocaffeine are the only purines which react as described with haem, CO-protohaem, porphyrins and metalloporphyrins. No other purine, out of about 20 tested, including theobromine, theophylline and 1:3:7-trimethyl uric acid, gives any of these reactions.

8. The fact that chlorocaffeine behaves in this respect like caffeine, shows that the H in position 8 can be replaced by Cl but not by O_2.

9. Of all the iminazole compounds tested, only pilocarpine, and to a lesser degree 1-methyl iminazole, react with the porphyrin in the same way as caffeine; but, unlike caffeine, with haematin, haem and CO-haem, they give parahaematin, haemochromogen and CO-haemochromogen compounds respectively.

10. The fact that globin and certain serum proteins react with porphyrins in the same manner as caffeine, while none of their amino-acid constituents, including histidine, gives this reaction, suggests that (*a*) either these proteins contain an additional, not yet isolated, constituent having these properties, or (*b*) that the iminazole ring of histidine, when the latter is incorporated in a polypeptide chain, may have some of the properties of the iminazole ring of caffeine and of pilocarpine.

11. The bearing of all these properties of caffeine on the mechanism of caffeine diuresis is discussed.

I wish to thank Prof. D. Keilin and Dr R. Hill for their helpful suggestions and interest in the progress of this work; Dr E. F. Hartree for the preparation of pure samples of iminazole, 1-methyl iminazole and methyl benziminazole; Dr P. Eggleton for samples of carnosine and anserine; and Dr F. G. Mann for samples of paraxanthine and heteroxanthine

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The Action of Proteolytic Enzymes on Fibrinogen Solutions

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It has been recognized for some years that a proteolytic enzyme system may be concerned with blood coagulation. Wright [1915] noted that blood coagulation could be inhibited by the addition of trypsin, and Douglas & Colebrook [1916] confirmed this observation, but demonstrated that smaller quantities of trypsin had the effect of *shortening* the clotting time.

Further studies of the effect of trypsin on blood coagulation have been reported by Tyson & West [1937], Eagle & Harris [1937], and Mellanby & Pratt [1938]. Though differences are evident between these investigations all the authors agree that trypsin in sufficient amounts prevents coagulation, and assume that the fibrinogen is destroyed. More recently, Tagnon, Davidson & Taylor [1942] have demonstrated the presence in chloroform-treated serum of a proteolytic enzyme which appears to be derived from the plasma globulins. This enzyme, like trypsin, prevents coagulation in large amounts, while in smaller amounts it hastens coagulation with subsequent digestion of the clot.

The work reported here is concerned mainly with the effect of pepsin on fibrinogen solutions and on plasma at *p*H values of 5·0–5·6. This limited *p*H range is necessary because at values below 5·0 fibrinogen tends to precipitate, and at values higher than 5·6 pepsin is inactivated.

The results indicate that under suitable conditions pepsin reacts immediately with fibrinogen, the product differing from fibrinogen in not being precipitable by 20% saturation with $(NH_4)_2SO_4$, or by half-saturation with NaCl. The fibrinogen modified by peptic action is referred to in this paper as 'non-precipitable fibrinogen' (i.e. not precipitable by low concentrations of salts), as compared with untreated fibrinogen, which is referred to as 'precipitable fibrinogen'.

METHODS

Plasma and serum. Horse plasma (oxalated and centrifuged) or serum was used, in most cases from horses which had not responded well to diphtheria immunization.

Solutions of fibrinogen. To 1000 ml. of plasma were added 300 ml. of sat. $(NH_4)_2SO_4$. The precipitate was collected by centrifuging, and dissolved in 1000 ml. of saline. The precipitation and solution were both repeated twice [cf. Warner, Brinkhous & Smith, 1935–6]. The final solution was adjusted to *p*H 5·4 by cautiously adding acetate buffer (*p*H 5·0) and was centrifuged before use.

'*Chloroform serum*' was prepared as described by Tagnon [1942].

Pepsin. This was a commercial (1/3000 U.S.P.) product.

p*H values* were determined by means of a glass electrode.

Estimation of salt-precipitable fibrinogen. Salt-precipitable fibrinogen in the plasmas and digest mixtures was assayed approximately by precipitation with 20% sat. $(NH_4)_2SO_4$, and collection of the precipitate by centrifugation. The precipitate was dried at room temperature by rolling on a No. 50 Whatman filter paper, was treated with ethanol for 5 min. and finally dried at 110° to constant weight. Though the presence of foreign material (e.g. $(NH_4)_2SO_4$) was unavoidable in the dried precipitate, the method in our hands proved sufficiently accurate for the purpose for which it is used in this work.

Estimation of heat-precipitable fibrinogen. Normal fibrinogen is precipitated by heating in solution to 56° for 2 min. For the estimation of heat-precipitable fibrinogen the precipitate was collected by centrifuging and dried as for salt-precipitable fibrinogen.

Temperature of enzyme-fibrinogen mixtures. Throughout this work the enzyme-fibrinogen mixtures were made up at room temperature (18°).

RESULTS

The effect of pepsin on plasma

In order to determine the amount of pepsin which would render non-precipitable the fibrinogen in a given quantity of plasma, both pepsin and plasma were adjusted to *p*H 5·4. To 10 ml. quantities of plasma in test tubes was added a 1% pepsin solution, beginning with 1 ml. in the first tube and finishing with 10 ml. in the last. All volumes were adjusted to 20 ml. with water. 2 ml. quantities were removed from the tubes at intervals and these were tested for fibrinogen by the addition of 0·5 ml. of sat. $(NH_4)_2SO_4$ solution. This range usually included the end-point, giving a precipitate in the tubes with the smaller, but none in those with larger, amounts of pepsin. The end-point changes little after the first 5 min. during periods up to 24 hr. The results of a typical experiment are given in Table 1.

Table 1. *The reaction between plasma fibrinogen and 1% pepsin at* p*H 5·4 and 18°*

Time from beginning of exp. min.	ml. pepsin/10 ml. plasma				
	1	2	3	4	5
8	+ + +	+ + +	+ +	+ +	+ +
30	+ + +	+ + +	+ +	+ +	+ +
120	+ + +	+ + +	+	+	+
840	+ + +	+ +	+	+	–
	ml. pepsin/10 ml. plasma				
	6	7	8	9	10
8	+ –	+ –	–	–	–
30	–	–	–	–	–
120	–	–	–	–	–
840	–	–	–	–	–

The data indicate the precipitates obtained on the addition of $(NH_4)_2SO_4$ to 20% saturation.

+ + + = heavy precipitate, + + = moderate precipitate, + = light precipitate, + – = opalescence, – = no precipitate.

In order to render the tests quantitative, larger digest mixtures were prepared corresponding to selected tubes which, in the preliminary test, had precipitated with 20% sat. $(NH_4)_2SO_4$. Usually 10 or 20 times as much of each reagent was employed in the larger digest mixtures. 100 ml. of the digest were removed at 5 and 15 min. after mixing, and added to 25 ml. of sat. $(NH_4)_2SO_4$ solution. A control experiment was done with plasma and water only. The results obtained in a typical experiment are given in Table 2.

Table 2. *The effect of increasing amounts of pepsin on the amount of fibrinogen precipitable from plasma by 20% saturation with* $(NH_4)_2SO_4$ *at* p*H 5·4 and 18°*

ml. of 2% pepsin/200 ml. plasma	Fibrinogen precipitable from 100 ml. of digest mixture (g.)	
	After 5 min.	After 15 min.
0	0·2458	—
20	0·1498	0·1500
40	0·064	0·044
60	Nil	Nil

Vol. of each digest mixture was made up to 400 ml. with H_2O.

The effect of pepsin on solutions of fibrinogen

Solutions of fibrinogen were treated in a similar manner to the plasma. In a typical experiment tests showed that between 1 and 1·2 ml. of 0·2 % pepsin prevented the precipitation, by $(NH_4)_2SO_4$, of the fibrinogen in 5 ml. of solution. Having determined these approximate values larger digest mixtures were prepared. After standing at room temperature for 10 min., the precipitable fibrinogen was estimated in 100 ml. of each mixture. The results are given in Table 3.

Table 3. *The effect of increasing amounts of pepsin on the amount of fibrinogen precipitable from fibrinogen solutions by 20 % saturation with* $(NH_4)_2SO_4$ *at* p*H 5·4 and 18°*

ml. of 0·2 % pepsin/150 ml. of fibrinogen solution	Fibrinogen precipitable from 100 ml. of digest mixture after 10 min. (g.)
0	0·2855
3	0·2210
9	0·1315
21	0·0285

Vol. of each digest mixture was made up to 210 ml. with H_2O.

The results in Tables 2 and 3 indicate that fibrinogen in plasma requires more enzyme per g. to render it non-precipitable than that in solutions of fibrinogen prepared by our method.

A comparison of the amount of fibrinogen precipitable from pepsin-treated plasma by $(NH_4)_2SO_4$ *and by heat in the presence of active and alkali-inactivated enzyme*

In order to furnish data as to the fate of the fibrinogen on pepsin treatment, the effect of heat precipitation of the fibrinogen in pepsin-treated plasma was compared with that of salt precipitation. Experiments were carried out at *p*H 5·4, at which pepsin is comparatively stable, and at *p*H 8·2, at which pepsin is inactivated [Northrop, 1930].

Three digest mixtures were prepared by adding to three 200 ml. quantities of plasma at *p*H 5·4 80, 120 and 160 ml. of 1 % pepsin. The volumes of all the mixtures were adjusted to 400 ml. with water.

Estimation of the salt-precipitable fibrinogen. (*a*) *At* p*H 5·4.* 100 ml. quantities of the above mixtures were removed and the salt-precipitable fibrinogen estimated.

(*b*) *At* p*H 8·2.* Only the mixture containing the greatest amount of pepsin, and therefore the least amount of salt-precipitable fibrinogen, was studied. 100 ml. of this were removed, its *p*H was adjusted to 8·2, and it was allowed to stand at room temperature for 3½ hr., before estimation of the precipitable fibrinogen. This time interval was necessary since the non-precipitable fibrinogen was not immediately rendered precipitable on raising the *p*H to 8·2.

Estimation of heat-precipitable fibrinogen. (*a*) *At* p*H 5·4.* A 100 ml. quantity of the mixture containing the largest amount of pepsin was heated at 56° for 2 min.

(*b*) *At* p*H 8·2.* 100 ml. quantities of all three mixtures were adjusted to *p*H 8·2, and heated at 56° for 2 min.

The weights of the fibrinogen precipitates obtained under the conditions given above are shown in Table 4.

Table 4. *A comparison of the amounts of fibrinogen precipitable from digest mixtures by heat and by salt at* p*H 5·4, and at* p*H 8·2*

Volume of 1 % pepsin solution/ 200 ml. plasma ml.	Precipitate with $(NH_4)_2SO_4$ from 100 ml. of mixture g.		Precipitate by heating at 56° for 2 min. from 100 ml. of mixture g.	
	At *p*H 5·4	At *p*H 8·2	At *p*H 5·4	At *p*H 8·2
160	0·0472	0·2292	0·2498	0·2968
120	0·1608	—	—	0·2928
80	0·2648	—	—	0·2838
0	0·2808	—	—	0·2872

The effect of pepsin treatment on the clotting of plasma

Reference has already been made to the work of other investigators on the effect of active trypsin on blood coagulation. The effect of a proteolytic enzyme on plasma coagulation in the presence of active enzyme, and also following inactivation of the enzyme by mild alkali treatment, has now been studied. To 35 ml. of plasma were added 45 ml. of 1 % pepsin. The solution after 2 min. gave no precipitate in 20 % sat. $(NH_4)_2SO_4$. The *p*H of the mixture was then raised to 8·2. 0·1 ml. of $CaCl_2$ solution (sp. gr. 1·2) was added to 10 ml. of the mixture, which gave a clot after incubation at 37° for 25 min. The addition of 5 ml. of normal horse serum to 10 ml. of the mixture to which $CaCl_2$ had been added, gave a clot in less than 5 min. at 37°.

Efforts to clot plasma at *p*H values of 5·0–5·6 in the presence of active pepsin have failed consistently, whereas after inactivation of the pepsin by alkali, clots have been obtained at *p*H values of 5·5, 6·2 and 7·2.

Experiments with trypsin, 'chloroform serum' and papain

The treatment of plasma with trypsin (B.D.H.) at *p*H 8·0 showed that at room temperature this enzyme behaved similarly to pepsin, but since this enzyme, unlike pepsin, is not easily inactivated, no effort has been made in this case to determine whether the change produced is similar to that produced by pepsin. Other workers [Eagle & Harris, 1937], however, have declared that the fibrinogen is

destroyed by an excess of this enzyme, though it may be that in the efforts to prove its presence by heating, true digestion was brought about. Experiments have shown that at room temperature the amount of fibrinogen not precipitable by 20% saturation with $(NH_4)_2SO_4$ is approximately proportional to the amount of trypsin added. The reaction proceeds rapidly, and little difference is noted in the weight of fibrinogen precipitable at 1, 15 and 30 min.

An experiment with 'chloroform serum' at *p*H 6·0 showed that between 2 and 3 ml. of this would prevent the precipitation of fibrinogen in 1 ml. of plasma, whereas a control experiment with untreated serum gave a heavy precipitate of fibrinogen at all levels.

The results with papain at *p*H 5·6 were similar to those with trypsin.

DISCUSSION

The results above show that pepsin and fibrinogen react at *p*H values of 5·0–5·6 in such a way as to render the fibrinogen non-precipitable at 20% saturation with $(NH_4)_2SO_4$. The amount of fibrinogen rendered non-precipitable is roughly proportional to the amount of enzyme added, and after the initial reaction the amount of fibrinogen modified by the enzyme is not appreciably increased during a subsequent period of 30 min. Inactivation of the pepsin by alkali restores to the fibrinogen the property of being precipitated by 20% sat. $(NH_4)_2SO_4$.

The alteration in the fibrinogen may be reversible, since, after alkali destruction of the enzyme, clots have been obtained with fibrinogen solutions which, in the presence of the active enzyme, had given no precipitate with 20% sat. $(NH_4)_2SO_4$, or with 50% sat. NaCl. No clot was obtained in pepsin-treated fibrinogen solutions unless the enzyme was first inactivated by alkali.

The conclusion drawn from these observations is that the enzyme combines with (or is adsorbed by) the fibrinogen, protecting it against precipitation at low salt concentrations, and against coagulation in plasma on the addition of Ca^{++}.

Solutions of partially purified fibrinogen require less pepsin per g. fibrinogen to produce this change than does plasma. Tagnon [1942] showed that more 'chloroform serum' was necessary to prevent the 'lysis' of fibrinogen in plasma than in purer fibrinogen solutions, and hence assumed some antilytic substance in plasma. It has been found in the present investigation that with many plasmas a certain amount of pepsin must be added before any fibrinogen is protected against precipitation, indicating that more than one substance must be competing for the enzyme. A comparison of graphs obtained for three separate experiments with different plasmas, by plotting the amount of fibrinogen rendered non-precipitable by low concentrations of salts, under the influence of pepsin, against the amount of 1/3000 pepsin present, illustrates this point (Fig. 1).

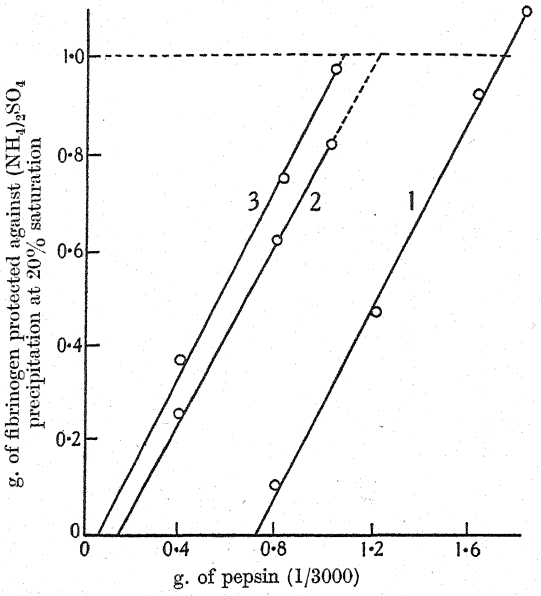

Fig. 1. The influence of the presence of pepsin on the precipitability of fibrinogen in solution by low concentrations of salts. Curves 1–3 represent different experiments with three different preparations of horse plasma.

The extension of the above work to other proteolytic enzymes is of interest in view of the discovery of Gorter, Maaskant & Campagne [1936] that trypsin has an explosive-like action on the spreading of fibrinogen in monomolecular films. Other authors have observed that trypsin and 'chloroform serum' can inhibit the coagulation of plasma, and the present work shows that trypsin and an enzyme present in 'chloroform serum' can protect fibrinogen against precipitation by low concentrations of salts. This raises the question as to whether the reaction between these enzymes and fibrinogen is similar to that between pepsin and fibrinogen, and whether inactivation of these enzymes would restore the power of clotting to solutions of fibrinogen which had been rendered non-coagulable in the presence of the active enzyme.

SUMMARY

1. Pepsin reacts with fibrinogen at *p*H 5·0–5·6 in such a way that the fibrinogen is rendered non-precipitable with 20% sat. $(NH_4)_2SO_4$ and non-

coagulable in the presence of plasma containing Ca^{++} ions.

2. Alkali destruction of the enzyme restores to the fibrinogen the power of forming clots under suitable conditions, and of being precipitated by low concentrations of salts.

3. Certain other proteolytic enzymes react similarly under suitable conditions.

I am indebted to the Medical Director of this Institute for providing facilities for carrying out these experiments, and to Mr L. N. Owen who has performed much of the experimental work.

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The Separation of Fibrinogen from 'Pepsin-inhibitor'

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Numerous methods for the purification of fibrinogen by precipitation with $(NH_4)_2SO_4$ or NaCl have been described [Florkin, 1930; Warner, Brinkhous & Smith, 1935–6; Ferguson, 1938–9; and Ferguson & Erickson, 1939]. Florkin [1930] used solubility in salt solutions as the criterion of purity of his preparations.

In a previous paper [Hind, 1943] it was shown that pepsin, when present in sufficient amounts, would protect fibrinogen against precipitation at low salt concentrations, and that a solution of partially purified fibrinogen required less enzyme per g. of fibrinogen to effect this protection than did fibrinogen present in plasma. In the present work we show that a substance (or substances) present in plasma competes with fibrinogen for the enzyme. The substance (or substances) having this inhibiting effect on pepsin is termed 'pepsin inhibitor' in the following account. Some properties of the pepsin inhibitor and its separation from fibrinogen are described.

METHODS

Horse plasma (centrifuged and oxalated) and serum were used.

Pepsin was a commercial (1/3000 U.S.P.) product.

Estimation of the salt-precipitable fibrinogen and the preliminary and semi-quantitative tests between pepsin and fibrinogen were carried out as previously described [Hind, 1943].

Purified fibrinogen. 1000 ml. of centrifuged plasma were precipitated with 21·6 % sat. $(NH_4)_2SO_4$. The precipitate was collected on the centrifuge and dissolved in 1000 ml. of saline. The precipitation and solution were repeated 4 times. On redissolving for the last time, however, only 500 ml. of saline were used.

Pepsin inhibitor and serum globulins. The residues obtained from the purified fibrinogen were precipitated with 50 % sat. $(NH_4)_2SO_4$ and the globulin precipitate was filtered off with kieselguhr, and pressed. The kieselguhr-protein mixture was then extracted 3 times with 200 ml. quantities of water and the solution dialysed overnight. The dialysed solution was made up to 1000 ml., i.e. the original volume of the plasma used.

Serum. Serum was prepared from 400 ml. of plasma, by adding $CaCl_2$ and pressing the clot. The serum gave no precipitate with 21·6 % sat. $(NH_4)_2SO_4$.

Heated serum was prepared by heating 150 ml. of oxalated serum to 58° for 2 min.

Defibrinated plasma. 400 ml. of plasma were heated to 56° for 2 min. and centrifuged until clear.

RESULTS

The effect of repeated precipitation of fibrinogen on its reaction with pepsin

1000 ml. of plasma were clarified by centrifuging at 5000 r.p.m. for 5 min., and 200 ml. were removed for subsequent tests. Solid $(NH_4)_2SO_4$ to 21·6 % saturation was dissolved in the remaining 800 ml. The fibrinogen precipitate was collected by centri-

fuging and redissolved in normal saline. It was made up to 800 ml. and 200 ml. were removed. The precipitation and solution were repeated twice more. The volume was made up in each case to correspond to the volume which had been precipitated, and at each stage 200 ml. of solution were removed.

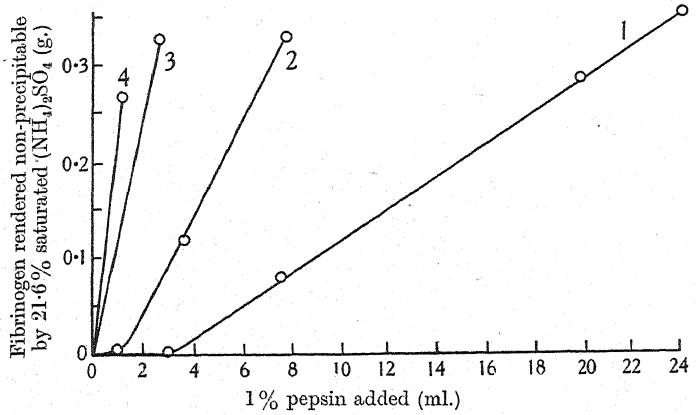

Fig. 1. Pepsin titration curves of fibrinogen solutions after successive reprecipitations. Curves 1–4 were obtained with plasma, once precipitated fibrinogen, twice precipitated fibrinogen and thrice precipitated fibrinogen respectively. The data plotted were obtained by subtracting the weight of fibrinogen precipitated in the presence of pepsin from that precipitated in its absence. This difference was plotted against the volume of 1% pepsin which had been added.

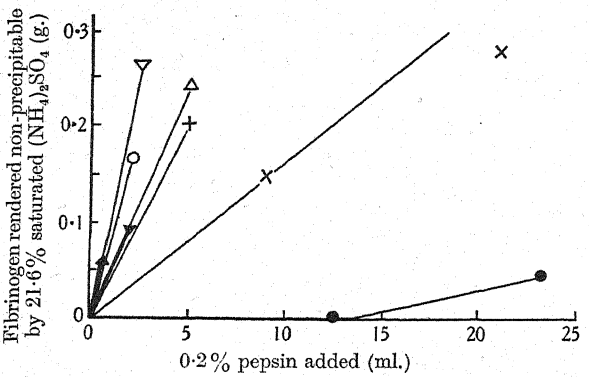

Fig. 2. Pepsin titration curves of fibrinogen solutions from different plasmas. ▽ Fibrinogen reprecipitated 3 times; ○ fibrinogen reprecipitated 3 times; △ fibrinogen reprecipitated 3 times; ▼ fibrinogen reprecipitated 4 times; ▲ fibrinogen reprecipitated 3 times; + fibrinogen reprecipitated 4 times; × fibrinogen reprecipitated twice; ● plasma (typical curve).

After the determination of the approximate values for the amount of pepsin required to prevent the salt precipitation of the fibrinogen in 2 ml. of each fraction, quantitative determinations were carried out. 50 ml. of solution of each fraction were used, and after treating with the predetermined amount of 1% pepsin for 10 min., the precipitable fibrinogen was estimated. The curves obtained from the results are given in Fig. 1. The weight of fibrinogen protected against precipitation represents the difference in weight between a control precipitate and the precipitate obtained from a pepsin-fibrinogen mixture. This difference in weight is plotted against the amount of pepsin which produced this change. It will be seen that at each successive stage of purification less enzyme was required to protect the fibrinogen against salt precipitation, and that at least 4 or 5 precipitations would be necessary to effect complete separation of the fibrinogen from the pepsin inhibitor.

Similar curves for fibrinogen preparations from different plasmas are given in Fig. 2.

Preliminary tests with the pepsin inhibitor

The effect of heat on the pepsin inhibitor in serum and in plasma was studied, and the pepsin inhibitor content of plasma was compared with that of serum prepared from the plasma.

Solutions used. (1) 200 ml. plasma + 100 ml. saline. (2) 150 ml. of solution of pepsin inhibitor and serum globulins + 75 ml. of fibrinogen solution. (3) 150 ml. of oxalated serum + 75 ml. of fibrinogen solution. (4) 150 ml. of heated serum + 75 ml. of fibrinogen solution. (5) 150 ml. of heat-defibrinated plasma + 75 ml. of fibrinogen solution. (6) Undiluted fibrinogen solution.

These mixtures were all adjusted to *p*H 5·3. The fibrinogen content of the solutions was comparable except that (6) contained 3 times as much fibrinogen as the other solutions.

Preliminary tests with 2 ml. quantities of the solutions were carried out against 1·0 and 0·01% pepsin. The lower strength was found necessary, as with some of the mixtures very little pepsin was required to render the fibrinogen non-precipitable. For quantitative determinations 50 ml. of solution were used and, after treatment with appropriate amounts of pepsin for 10 min. at 18°, the precipitable fibrinogen was estimated. The amount of pepsin (g.) required to protect 1 g. of fibrinogen in each of the solutions was calculated and is given in Table 1.

Further tests have shown that serum heated to 55° does not lose its ability to inhibit the action of pepsin in rendering fibrinogen non-precipitable.

Table 1. *The reaction at pH 5·3 between pepsin and different solutions containing fibrinogen*

Fibrinogen-containing solution used	Pepsin required to protect 1 g. of fibrinogen against $(NH_4)_2SO_4$ precipitation at 21·6% saturation g.
Plasma	0·9
Fibrinogen	0·02
Solution of fibrinogen and pepsin inhibitor	1·05
Fibrinogen and serum	1·8
Fibrinogen and heated serum	0·06
Fibrinogen and heated defibrinated plasma	0·08

DISCUSSION

Because of the close association between the pepsin inhibitor and fibrinogen, and because fibrinogen solutions could be approximately titrated against pepsin, it was decided to use the pepsin-fibrinogen reaction as a measure of the purity of fibrinogen solutions. The results show that four or five reprecipitations with 21·6 % sat. $(NH_4)_2SO_4$ are necessary to separate the pepsin-inhibitor from fibrinogen. The concentration of $(NH_4)_2SO_4$ (21·6 % sat.) used in this work was determined by preliminary experiments to be a safe minimum quantity for the precipitation of the fibrinogen from the plasma used. Most other workers have used 25 % saturation. 1 g. of pepsin (1/3000 U.S.P.) will protect approximately 50 g. of our purest preparations of fibrinogen against precipitation with $(NH_4)_2SO_4$. Similar values have been obtained by using fibrinogen solutions prepared from different plasmas.

Little or no pepsin inhibitor could be demonstrated in plasma defibrinated by heating, or in serum which had been heated to 58°. However, the pepsin inhibitor is apparently stable at 55°, since serum heated to this temperature showed no loss of potency. It was present, together with the serum globulins, in the filtrates obtained during the preparation of fibrinogen, and was precipitated therefrom by precipitating with 50 % sat. $(NH_4)_2SO_4$. On adding this precipitate to a purified fibrinogen solution, the reaction between the mixture and pepsin was similar to that between plasma and pepsin. This pepsin-inhibitory action of serum appeared to be greater than that in the plasma from which the serum had been prepared.

SUMMARY

1. A substance ('pepsin-inhibitor') is present in fresh serum and plasma, which inhibits the ability of pepsin to protect fibrinogen against precipitation at *p*H 5·0–5·6 by low concentrations of salt.

2. This pepsin inhibitor is present in preparations of fibrinogen precipitated with 20–25 % sat. $(NH_4)_2SO_4$, but separation of it from fibrinogen can be effected by repeated precipitation of the latter with 21·6 % sat. $(NH_4)_2SO_4$.

3. Heating at 58° for 2 min. destroys almost all the activity of preparations of this 'pepsin-inhibitor'.

I am indebted to the Medical Director of this Institute for providing facilities for carrying out these experiments and to Mr L. N. Owen who has performed much of the experimental work.

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Microdetermination of Vitamin A and Carotenes

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The Carr-Price reaction has been widely used for the estimation of vitamin A and carotenes. The limitations and difficulties of the method have been described by so many that no attempt will be made to review the extensive literature [see Brode & Magill, 1931]. The conventional method, as used by May, Blackfan, McCreary & Allen [1940] and by Dann & Evelyn [1938] for the determination of β-carotene by measuring the light absorption with a 440mμ filter, is not specific. For example, in blood serum both bilirubin and β-carotene absorb light of wave-length 440mμ.

The optical method described here was developed to overcome the difficulties in the quantitative estimation of the free* vitamin A. The application of the same method for the accurate determination of β-carotene has been studied. The instrument is an adaptation of a photoelectric colorimeter developed by Yntema & Fleetwood [1937]. It differs from it in one fundamental respect. A beam of light after having traversed the sample (Carr-Price reaction) is split into two beams which pass through 620 and 589 mμ filters, respectively, before reaching two recording photocells. Thus a direct simultaneous and mutually independent determination of vitamin A and carotene in one and the same sample can be achieved, providing that (1) the beams are monochromatic, or nearly monochromatic; (2) the temperature is close to 0° (cold room). On the other hand, only vitamin A can be determined with this apparatus at room temperature, because the 589 mμ band of β-carotene fades within a few seconds.

EXPERIMENTAL

The apparatus used for measuring the intensity of the blue colour produced by the reaction of vitamin A with $SbCl_3$ is shown diagrammatically in Fig. 1. The accessories are described in the diagram.

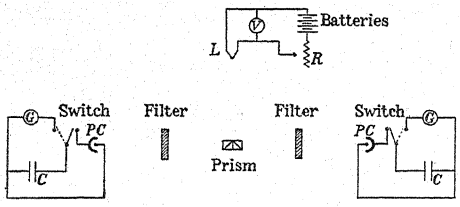

Fig. 1. Circuit for vitamin A: batteries, 6 V.; lamp, 100 c.p. 6–8 V.; rheostat, 0·5 ohm.; voltmeter, 0–8; photocells, RCA 922. Circuit for β carotene: galvanometers, L-N R 2500 *f*; condensers, L-N 1 μF.; switches, spot; filter, Pyrex 625 mμ no. 245; filter, 589 mm.

The light source was a Mazda lamp, 100 c.p., 6–8 V., supplied by batteries, constant voltage being maintained by means of a rheostat and voltmeter. A glass-stoppered cell, 1 ml. in capacity and 5 cm. long, as shown in Fig. 2, contained the solutions for the Carr-Price reaction. This cell was in position between the light source and a set of two prisms which served to divide the light which had traversed the solution into two beams, one beam passing through the 620 mμ filter to one recording photocell for vitamin A, and the other beam (not used in this investigation) passing through the 589 mμ filter to the other photocell which measures β-carotene. The current generated by the influence of light upon the photoelectric cells is stored in the condensers and is measured by determining the sudden deflexion induced by discharging these through a ballistic galvanometer.

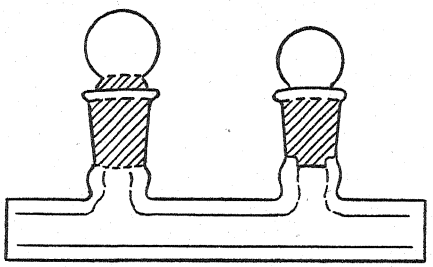

Fig. 2.

The procedure for the determination of vitamin A at room temperature was standardized as follows. A vitamin A concentrate containing 200,000 U.S.P. units/g. was dissolved in chloroform (reagent quality) so that 0·5 ml. contained from 10 to 100 U.S.P. units. To the cell (Fig. 2), 0·5 ml. of the standard containing a known amount of vitamin A was added. To this was added 0·5 ml. of a saturated solution of $SbCl_3$ in $CHCl_3$ to develop the colour. Just as the solution of the latter was released from the pipette, a stop-watch was started. The contents of the cell were thoroughly mixed and the cell was placed in the receiver. After exactly ½ min. the light was turned on at a potential of 5 V. and allowed to pass through the solution in the cell for precisely 1½ min.; then the current stored in the condenser was determined by measuring the extent of the galvanometer deflexion. Repetition with varying amounts of vitamin A, from 0 to 100 U.S.P. units, and the plotting of the logarithms of the galvanometer deflexions against the U.S.P. units of vitamin A, resulted in a straight line (Fig. 3). To determine the vitamin A content of an unknown material dissolved in $CHCl_3$, the above procedure was followed. From the logarithm of the galvano-

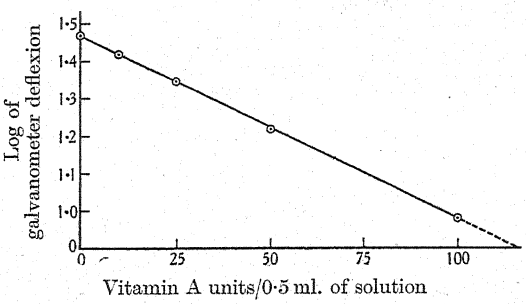

Fig. 3.

* The term 'free vitamin A' as here used refers to vitamin A alcohol, or vitamin A ester. We have some evidence that vitamin A may be found in a compound with other carotenoids.

meter deflexion, the amount of vitamin A was easily read off the chart.

Since the absorption band at 589mμ for β-carotene persists for only 5–10 sec. at room temperature, it is impossible to estimate this substance by measuring the density of the 589mμ band at 20–25°. However, we found that if the Carr-Price reaction is carried out in a cold room at 0°, the 589mμ band could be observed for a period of more than 2 min. when 2–4 vol. $SbCl_3$ (sat. at 0°) were added to 1 vol. β-carotene. The period during which the 620mμ band for vitamin A is observable can also be prolonged at the same temperature. Ender [1932] has shown that the substance responsible for the blue colour produced by the reaction of $SbCl_3$ with carotenoids is more stable at lower temperatures.

The advantages and accuracy of this method over the visual and photoelectric colorimetric methods are obvious. The difficulty of measuring precisely the maximum of the absorption point for blue colour produced by vitamin A or β-carotene with $SbCl_3$ is obviated, and the interference by other carotenoids is eliminated by using appropriate filters. The method would serve admirably for the estimation of small amounts of vitamin A and β-carotene in blood, subject to the temperature limitations discussed above; it is about 50 times as sensitive as the one of Dann & Evelyn [1938]; at 0° it is perhaps over 100 times as sensitive.

Since the cells used for the Carr-Price reaction have a tendency to develop, with use, a thin light-absorbing film of the hydrolysis products of $SbCl_3$, it is imperative that the cells be rigorously cleaned after every determination as follows: the cells* and stoppers were immersed in strong HCl for several minutes. After removing excess HCl with tap water, the cells were allowed to stand in chromic acid cleaning solution for 10 min. This was followed by thorough rinsing with tap water followed by distilled water, after which the cells were dried at 100–105° for $\frac{1}{2}$ hr.

The method has been used for the estimation of vitamin A in mouse and rat livers. The livers were saponified and extracted with peroxide-free ether according to the procedure of Davies [1933]. Spectroscopically the Carr-Price reaction with the unsaponifiable fraction of mouse livers gave only one visible absorption band, that at 620–625mμ.

The $CHCl_3$ solution of the unsaponifiable fraction of rat livers showed two absorption bands when treated with $SbCl_3$, one at 620mμ and the other at 570mμ. However, after 30 sec., at which time the determinations were made, no interference with the measurement of the density of the 620mμ band was observed spectroscopically. For the determination of vitamin A in the unsaponifiable fraction, or in the fat-soluble fraction of tissues or other substances, it was first necessary to make a spectroscopic examination of the Carr-Price reaction so as to determine whether there was any interference with the measurement of the density of the 620mμ band by other carotenoids. In our experiments this precaution was necessary because a glass filter (Corning 246 R.M. 3·5 mm.) transmitting a band in the red region of the spectrum, rather than a source of monochromatic light, was used.

To follow changes in the hepatic vitamin A content of rats, two groups of animals received intraperitoneal injections at regular intervals of methylcholanthrene and 3:4-benzpyrene dissolved in lard. (Goerner & Goerner [1939] and Baumann, Foster & Lavik [1941] have shown that carcinogens cause a lowering of the hepatic vitamin A content of rats.)

During the treatment some of the rats were killed and the vitamin A content was determined with 0·5 ml., or with a 1/50th part, of the $CHCl_3$ solution of the unsaponifiable fraction. The marked depletion of the liver stores of the vitamin are shown in Table 1.

Table 1. *Effect of carcinogens on hepatic vitamin A*

Animal no.	Total dose of hydrocarbon mg.	Vitamin A/ 0·5 ml. aliquot U.S.P. units	Vitamin A/g. liver U.S.P. units
N 1	0	32	106
N 2	0	56	249
N 3	0	40	160
N 4	0	48	247
MC 1	16	13	73
MC 2	16	2	16
MC 3	16	0	0
BP 1	16	3	13
BP 2	16	5	24
BP 3	16	12	68

Animals marked N represent the normal untreated animals; MC, methylcholanthrene treated; BP, benzpyrene treated.

The determination of 2–15 U.S.P. units in 0·5 ml. of solution demonstrates the micro nature of the method. The maximum error in the determinations of 10–100 U.S.P. units was 3%. It should not be difficult to develop procedures for the use of this method to determine vitamin A at room temperature for other tissues, or for both vitamin A and β-carotene at 0°.

SUMMARY

A micro-method for the estimation of vitamin A is described. Under proper conditions it can be used for the simultaneous and mutually independent

* The cells were made by the American Instrument Co., Silver Spring, Maryland. The end-plates of each cell were fused to the body of the cell so that cleaning could be carried out in chromic acid cleaning solution.

determination of vitamin A and β-carotene in the same sample. The application of the method for the determination of vitamin A in mouse and rat livers is briefly discussed.

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The Microbiological Assay of Riboflavin. The Influence of Inorganic Constituents and Unknown Growth Factors

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We are here concerned with the assay of riboflavin which has developed from the investigations of Snell & Strong [1939] and which showed the linear relationship between the acid production from glucose by *Lactobacillus casei* ε (Helveticus) and the concentration of riboflavin present in the medium. In this paper the effect of the addition of various inorganic constituents on the microbiological assay of riboflavin will be discussed, and suggestions made for the improvement of the method.

EXPERIMENTAL

The basal medium of Snell & Strong [1939] has been modified a number of times, notably by Snell & Wright [1941], Hutchings, Peterson & Shull [1942], and Lindner & Pollack [1942], mainly by the addition of substances which might function as growth factors or accessories. In our view the only real source of variation in the preparation of this medium is in the composition of the acid hydrolysate of casein. We have invariably prepared our hydrolysate according to the method used in J. H. Mueller's laboratory and described as Method 1 [Johnson & Mueller, 1941]. This hydrolysate is practically free from Fe and P and there is little, if any, Ca present.

The results which follow were obtained with a 20 % acid digest of casein, whose free HCl, when neutralized, gives 3·44 g. NaCl/100 ml. (1·72 g. NaCl/l. of medium). It contains 19·46 g. N_2/l., no P, and the Fe content is very small.

The basal medium first used was as follows: 50 ml. casein hydrolysate (20 %); 2 ml. 20 % cystine solution in 2·5*N* HCl; 0·1 g. l-tryptophan; 10·0 g. glucose; 0·5 g. KH_2PO_4; 100 μg. nicotinic acid; 50 μg. pantothenic acid; 50 μg. *p*-amino-benzoic acid; 2·5 ml. liver eluate; 0·4 mg. $FeSO_4.7H_2O$; 2·0 ml. mineral solution.

This was adjusted to *p*H 6·8 and the volume made up to 1 l.; the medium was sterilized for 10 min. at 10 lb./sq. in.

The mineral solution contained: 22·5 g. $MgSO_4.7H_2O$; 5·0 ml. $CuSO_4.5H_2O$ (1 % w/v); 4·0 ml. $ZnSO_4.7H_2O$ (1 % w/v); 1·5 ml. $MnCl_2.4H_2O$ (1 % w/v); 3·0 ml. conc. HCl; H_2O to 100 ml.

After sterilization in 20 ml. amounts in 125 ml. Pyrex flasks, riboflavin, or extracts of the food to be tested, were added in variable amounts and also 0·2 ml. of 0·5 % (w/v) $CaCl_2$.

The $CaCl_2$ was added since it was soon observed that in the absence of such addition, growth and acid production were negligible.

It will be noticed that the medium contains *p*-amino-benzoic acid. In the examination of the partially purified liver fraction it became apparent that this compound was present in the active material. Tests revealed the fact that the compound was a growth stimulant for the Lactobacillus and it was consequently included in the basal medium.

Preparation of active liver extracts

In early assay methods [Snell & Strong, 1939] photolysed peptone and yeast extract were added to the food medium; subsequently it was suggested that nicotinic acid and pantothenic acid could replace the yeast extract. In our experience this was

unsatisfactory; growth and acid production sometimes occurred, but the latter was so far short of an optimum that it was clear that additions made to a medium, when extracts of food or tissue fluids were added, modified the results. Because of deficiencies in source of supply, irregularity of result, etc., we began to use liver fractions which we were using in our studies on the nutrition of *C. diphtheriae* types. The properties of these active fractions have been described by Chattaway, Happold, Lythgoe, Sandford & Todd [1942]. Residues from the preparation of the liver anti-anaemic principle were adsorbed on Fuller's earth at *p*H 3, the bulk of the activity being in the filtrate. This was next adsorbed on norite. Activity was mainly found on the charcoal which was eluted with dilute NaOH until the eluate was slightly alkaline (*p*H 7·2) when elution was continued with water. After further acidification to *p*H 3, the eluate was extracted 5–8 times with amyl alcohol (in which the active component was insoluble). The aqueous phase was then concentrated and used instead of the yeast extract. It was free from riboflavin, and from all but traces of pantothenic and nicotinic acids. It should be noted that activity decreases on standing in weak solutions and this accounts for differences in total acidity in the series presented. The optimum activity of such a concentrate having been determined, it was added to the basal medium in the appropriate amounts.

RESULTS

Effect of Fe concentration on acid production

Twenty-four flasks each containing 20 ml. of medium were prepared in four series of 6. Series 1 contained no added $FeSO_4.7H_2O$, series 2 had 4 μg., series 3, 8 μg. and series 4, 16 μg. $FeSO_4.7H_2O$ added to each 20 ml. medium.

The six flasks in each series had 0, 0·2, 0·4, 0·6, 0·8 and 1·0 μg. riboflavin added respectively; 0·2 ml. 0·5% solution $CaCl_2$ was added to each flask. On incubation for 72 hr. the results in Table 1 were obtained.

Table 1. *Influence of addition to the medium of Fe and riboflavin on acid production*

Riboflavin added μg.	Acid production (ml. *N*/10)			
	$FeSO_4.7H_2O$ added			
	None	4 μg.	8 μg.	16 μg.
Nil	Nil	Nil	Nil	Nil
0·2	6·0	6·4	8·1	3·5
0·4	8·8	10·1	10·1	4·1
0·6	11·5	11·6	11·7	6·4
0·8	11·0	11·9	12·3	9·6
1·0	3·5	9·4	9·8	7·5

It will be observed that the greatest acid production was with 8 μg. $FeSO_4.7H_2O$/20 ml. but that the straight-line relationship which existed up to 0·6 μg. riboflavin at this level was extended up to the 0·8 μg. level with the higher concentration of the ferrous salt. It is not suggested that these levels will be the same for each batch of medium and they are almost certainly affected by the concentration of iron added in the liver eluate, but it is clear that the amount of Fe added with any food or tissue extract which is being assayed for riboflavin content should be known and suitable addition of Fe salt made to the control riboflavin series. Fortunately, tables of the mineral contents of foods and tissues are available and suitable additions can be made. That this is important will be realized by comparison of the above figures where 0·2 μg. in series 1 or 2 is approximately equal to 0·6 μg. riboflavin in series 4. Examination of the actual curves makes other differences apparent.

A further series of four flasks containing 0·8 μg. riboflavin and 5, 10, 15, 30 μg. $FeSO_4.7H_2O$ was tested (Table 2).

Table 2. *Influence of addition of Fe on acid production*

$FeSO_4.7H_2O$ μg.	Acid produced (ml. *N*/10) in		
	72 hr.	96 hr.	120 hr.
5	3·30	12·70	22·80
10	3·30	12·40	22·50
15	2·80	11·50	20·70
30	2·50	11·10	16·60

The Ca concentration in this experiment was double that used in the first, and, as will be seen later, was nearer to the optimum. These results agree well with those of the first series in showing a decrease in acid production with increasing concentration of $FeSO_4.7H_2O$.

In a further series in which $FeSO_4.7H_2O$ was added to the optimum of 400 μg./l., in 250 μg., 500 μg., 1 mg., 2 mg., 3 mg., 4 mg., and 5 mg. amounts, there was a decrease in acid formation down to the 3 mg. level, after which stabilization occurred. Thus the higher level of Fe in the Snell-Strong medium is sound for assay work, provided that it be realized that this medium does not give optimum acid production.

The effect of variations in $PO_4^{\equiv}$ and K^+ content of the medium

In the Snell-Strong medium $PO_4^{\equiv}$ is added as KH_2PO_4 (0·5 g./l.). This is the only source of K^+ and of phosphate in the medium. Since the importance of $PO_4^{\equiv}$ and of K^+ in carbohydrate metabolism is obvious, variations in their concentration have been tested. The first series of results presented below is with two separate fractions of liver extract; those appearing beneath the horizontal dividing line represent tests carried out some

weeks later than those above. The later tests contain 0·02 g. KH_2PO_4/20 ml. medium; 0·25 ml. 0·5 % (w/v) $CaCl_2$ solution was added to each 20 ml. medium after sterilization. This concentration of $CaCl_2$ does not represent an optimum. In the first series of results the optimum was obtained with a 0·1 % (w/v) KH_2PO_4 concentration. Addition of further phosphate as the Na salt was without effect with fraction 'H', but with fraction 'S' there was a rise over a range of addition from 0·13 to 0·016 g. %. Fraction 'S' was obviously more active than fraction 'H' in encouraging growth, so that the phosphate present as the K salt was adequate with 'H', whereas with 'S' the further addition of Na phosphate to *M*/800 was necessary to give optimal results. It is encouraging that 8 times this concentration did not affect the result at 5 days, since this suggested that in assay work increased concentration of $PO_4^{\equiv}$ due to that added with the material to be assayed, would not affect the result to an important degree (Table 3).

Table 3. *Influence of addition of phosphate on acid production from 20 ml. medium containing 0·8 μg. riboflavin*

KH_2PO_4 added g.	Medium + liver fraction 'S'. Acid produced (ml. *N*/10) in 72 hr.	96 hr.	120 hr.	Medium + liver fraction 'H'. Acid produced (ml. *N*/10) in 72 hr.	96 hr.
None					
+0·02	7·30	15·30	—	3·70	6·10
0·01	7·20	11·55	—	3·00	5·90
0·005	5·60	7·60	—	2·90	5·30
0·02	4·00	5·80	16·80	3·10	6·20
0·02 + Na phosphate buffer *p*H 7·2 to					
M/100	3·70	7·40	22·80	—	—
M/200	5·80	10·00	23.00	3·60	6·30
M/400	6·20	10·20	23·20	3·20	6·20
M/800	8·00	12·00	22·30	2·90	6·20

At this stage the concentration of KH_2PO_4 in the basal medium was doubled (0·04 g./20 ml. or 0·2 %); this should give a safe optimum for phosphate. There was a pronounced rise in acid production which was attributed to the increased K^+ concentration; actually this latter was not yet at its optimum (Table 4).

Table 4. *Influence of addition of KCl on acid production*

	Added KCl mg.	ml. *N*/10 acid produced in 48 hr.	66 hr.
Medium (20 ml.)		2·5	4·85
,,	17·0	3·0	5·30
,,	13·6	3·2	5·60
,,	10·2	2·9	5·30
,,	6·8	3·0	5·60
,,	3·4	3·2	5·50

This represented an additional K^+ optimum of 1·8 mg./20 ml. or 9 mg. %, though it is possible that for this medium it was less than this figure. The general stability of result with further increase in K^+ is again encouraging. Additions as KH_2PO_4 were also made and the results of a specimen experiment are given in Table 5. The optimum value was reached when the KH_2PO_4 was increased by 5 mg. KH_2PO_4/20 ml. or 25 mg./100 ml.

Table 5. *Influence of addition of KH_2PO_4 on acid production*

(Reaction adjusted with *N*/10 NaOH after addition of further phosphate. Medium tested in 20 ml. lots.)

	Added KH_2PO_4 mg.	Acid produced (ml. *N*/10) in 48 hr.	72 hr.	96 hr.
Basal medium (0·2 % KH_2PO_4)		1·90	4·0	8·5
,,	5	2·30	5·10	10·10
,,	11	2·25	4·90	9·90
,,	30	2·15	4·75	9·45
,,	50	2·20	4·85	9·65

The K^+ increase was thus 7·2 mg./100 ml. (9 mg. with KCl) and the total K^+ concentration added as KH_2PO_4 was 0·065 g./100 ml. Since the Na value (by neutralization of free HCl) was 0·079 g./100 ml., the K/Na ratio for additions was at least 0·82, a value which approximated to that obtained for optimal metabolism of stored carbohydrate in cells of *Escherichia coli* as shown by Evans, Handley & Happold [1942].

The effect of variation in the Ca^{++} concentration

The importance of this effect is easily observed in Table 6.

Table 6. *Influence of addition of $CaCl_2$ on acid production*

	Added 0·5 % $CaCl_2$ ml.	Acid (ml. *N*/10) produced/20 ml. medium in 72 hr.	96 hr.
Basal medium (0·2 % KH_2PO_4)		Nil	Nil
,,	0·1	6·35	16·10
,,	0·2	4·85	16·85
,,	0·3	12·05	23·45
,,	0·4	6·75	18·55
,,	0·6	4·00	15·40
,,	0·8	4·10	11·60

A further series with a different liver fraction gave a $CaCl_2$ optimum of 0·35 ml. of 0·5 % $CaCl_2$ solution. It will be noticed that there was a steep drop with excess of Ca. In view of the high concentration of Ca in milk it is important that the Ca curve for any medium which is being used to assay

milk products should be made and that the Ca equivalent of the milk should be added to the control riboflavin series in each assay. It will be noticed that each 0·1 ml. was equivalent to 0·18 mg. Ca^{++}. The Ca concentration of the medium before the addition of the $CaCl_2$ was 0·41 mg. Ca/100 ml., or 0·082 mg. Ca in each flask of test medium.

The effect of variation in the NaCl concentration

It was found that the basal medium prepared as described above did not contain an optimum concentration of NaCl. Preliminary experiments showed that the optimum lay between 90 and 100 mg. NaCl/20 ml. medium. The results (Table 7) show more exactly the value of this optimum concentration and also that addition of NaCl above this level does not cause a serious drop in acid production. Thus its addition in food or tissue extracts will not greatly affect riboflavin assay. All other mineral constituents and riboflavin itself (0·8 μg./20 ml.) were at an optimum for this experiment.

Table 7. *Influence of addition of NaCl on acid production*

NaCl/20 ml. medium mg.	ml. *N*/10 acid produced in 72 hr.
54 (basal medium)	1·9
89	2·25
91	2·65
93	3·6
94	3·2
95	2·9
97	2·7
99	2·7
134	2·7

Application of assay method

Assays were carried out on cabbage, new potatoes, meat and garden peas (served in British Restaurants), and though the Ca and K concentration of the media used was not at the optimum the results obtained with the first two foodstuffs illustrate the type of difficulty which may arise from the addition of unknown growth factors contained in a food extract to the basal medium. The extracts were prepared by refluxing the foodstuff for 2 hr. with an excess of *N*/2 HCl and then adjusting the reaction of the extract to *p*H 6·8. The extracts were added to two series of media which contained liver extract equivalent either to 12 g. or to 6 g. of raw liver. The results with the meat and the garden peas were useless because these contained either further growth factors, or more probably additional mineral constituents such as K, in significant amounts. The results with cabbage and new potato (Table 8), which do not add significant amounts of the important mineral constituents, are of interest in that they fit the curve of riboflavin variation at the higher concentration of liver extract but not at the lower one (Table 8 and Fig. 1).

It will be seen that the data of Table 8 fall on curve *A* (Fig. 1) rather than on curve *B*. The data derived from the addition of 0·5 ml. extract are probably less reliable than the other results in Table 8, because this dose of extract contained too little added riboflavin. We may therefore assume that cabbage contains about 41·5 g./100 g. and potato between 160 and 195 g. riboflavin/100 g., the latter figure probably being closer to the real value.

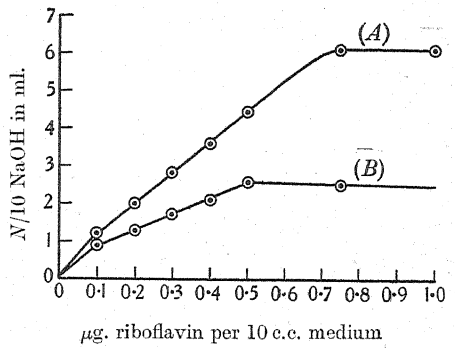

Fig. 1. Relationship between concentration of riboflavin present in the medium, and titratable acidity produced by growth of *Esch. coli*. (*A*) basal medium + liver extract equivalent to 12 g. liver. (*B*) basal medium + liver extract equivalent to 6 g. liver.

Table 8. *Influence of addition of cabbage and potato extract on acid production*

Material extracted	Material equiv. to 100 ml. extract g.	Extract added to medium ml.	Acid produced ml. *N*/10	Riboflavin content (by ref. to curve *A*, Fig. 1) of: Extract added to medium μg.	Extract added to medium μg. calc.*	100 g. of material μg.
Cabbage	42·2	2·0	3·2	0·35	—	41·5
		1·5	2·5	0·26	0·26	41·7
		1·0	1·6	0·15	0·17	35·5
Potato	18·5	1·0	3·3	0·36	—	195·0
		0·5	1·6	0·15	—	163·0

* Calculated on the assumption that the value for 2·0 ml. is correct.

The riboflavin content of the cabbage is probably 41·5–41·7 μg./100 g. raw cabbage, the potato is probably nearer the higher value of the two presented.

DISCUSSION

It is of interest that Gillespie & Rubbo [1940] reported that biotin was not essential for *Cl. acetobutylicum* whereas the reverse was claimed by Park & Wood [1942]. There are marked differences in the mineral constituents in the media used by these two sets of investigators; in general the mineral content of the latter workers was 1/10 that of Gillespie & Rubbo. Park & Wood's concentrations would certainly be suboptimal for the Lactobacillus and it is again interesting to note that they report an enhancement of growth with ammonium phosphate instead of with asparagine. Assuming that both sets of results are correct it would appear that a change in the mineral balance of a medium has enabled the organism to dispense with biotin. Though this may seem unlikely, it does suggest that the amount of a growth factor necessary for optimum action is profoundly affected by the mineral balance. This lends additional emphasis to the main theme of this paper.

It should be added that a series of additions have been made to the basal medium by a variety of workers. We have tested many of these compounds and found them inactive. Thus, adenine, guanine, uracil, thymine and adenylic acid were all tried separately and together, and found to be inactive. Since these components may be of natural origin it seems likely to us that their reported activity has been due to the adsorption on to the compound tested of small but adequate amounts of a growth factor or growth factors.

SUMMARY

Earlier work had revealed a number of irregularities in the application of the microbiological method of assay for riboflavin, using *Lactobacillus casei* ε. Evidence is presented to show that acid production can be altered by a variation in the concentrations of Fe^{++}, Ca^{++}, K^{+}, $PO_4^{\equiv}$ and NaCl in the basal medium. Optimum concentrations for these constituents have been demonstrated and in addition it was shown that further increases in Fe^{++}, K^{-}, $PO_4^{\equiv}$ and NaCl concentrations do not result in a great decrease in acid production. Further addition of Ca^{++}, however, results in a serious decrease. The necessity for, and the effect of, the addition of a liver eluate to the basal medium was shown. The bearing of these results on the assay of foodstuffs for riboflavin has been discussed.

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The State of Tyrosine in Egg Albumin and in Insulin as Determined by Spectrophotometric Titration

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Numerous studies on the reactions between protein and hydrogen and hydroxyl ions have yielded important information as to the number and character of ionizable groups in the protein molecule. The detailed interpretation of the titration curves is, however, in many cases ambiguous. The assumption is often implicitly made that the dissociation constants of the various polar groups in proteins are not very different from those found in amino-acids or peptides. In this general form, such an assumption cannot be correct. Above and below the isoelectric point (I.E.P.) the protein carries a net positive or negative charge and the number and distribution of these charges will greatly influence the ionization of different groups. A considerable displacement of dissociation constants can therefore be expected as the net valency of the protein increases. Near the I.E.P. the electrostatic effect

will be small, if the charge distribution is symmetrical. Investigations of the effect of changes of temperature [Wyman, 1939], of addition of formaldehyde [Kekwick & Cannan, 1936] or of iodination [Neuberger, 1934] have in some cases been of assistance in apportioning parts of titration curves to certain groups. But the overlapping of the titration ranges of different ionizable groups renders a quantitative evaluation of dissociation constants difficult, and sometimes impossible. It occurred to us that the ionization of the phenolic group of tyrosine in the protein might be investigated by a spectroscopic method, without interference from other groups ionizing in the same region. This would indicate how far charge effects in general can influence the intrinsic dissociation constants of the relevant amino-acids.

Most organic acids and bases which absorb light in the visible or ultra-violet region have absorption spectra which differ considerably from those shown by their respective anions or cations. *p*H measurements by indicators are based on such shifts in absorption in the visible spectrum. Similar changes in absorption spectra in the ultra-violet have been noted with phenol, and with phenol derivatives such as tyrosine [Stenström & Goldsmith, 1926], and the dissociation constants thus obtained agree well with the values established by other means [Flexsner, Hammett & Dingwall, 1935]. Proteins absorb ultra-violet light, the maximum absorption occurring with light of wave-length between 2750 and 2850 Å., and it has been assumed that the absorption above 2750 Å. is due to tyrosine and tryptophan [Holiday, 1936]. The spectrum of the latter amino-acid changes very little with *p*H, while the ionization of the phenolic group of tyrosine is accompanied by an increase of the molecular extinction coefficient, and a shift in the long wave maximum of about 200 Å. It was thought therefore that the change of the absorption spectrum of a protein with change of *p*H could be used to determine the *pK* of the phenolic group in the native protein molecule.

EXPERIMENTAL

The tyrosine and tryptophan samples used had been recrystallized several times; 3:5-diiodotyrosine was kindly supplied by Dr C. R. Harington. Insulin was a recrystallized sample obtained from Messrs Boots; we used two preparations of thrice recrystallized egg albumin which were kindly put at our disposal by Prof. A. C. Chibnall, and by Dr K. Bailey, respectively.

The water content of insulin was assumed to be 10% (personal communication from Prof. Chibnall); the concentrations of the egg albumin solutions were estimated by the Kjeldahl method, assuming a N content of 15·7%. All measurements, including the *p*H estimations (hydrogen electrode), were made at $20 \pm 2°$.

The optical measurements were made with a Hilger medium spectrograph and Spekker photometer, using a tungsten-steel spark as light source. The wave-length scale was checked against the iron spectrum, and the maximum error in the relevant region was found to be 5 Å.

RESULTS

The dissociation constant of the phenolic groups of tyrosine and 3:5-diiodotyrosine

The absorption curves for tyrosine and tryptophan obtained by us (Table 1) differ slightly from those reported by Holiday [1936] and Holiday & Ogston [1938]. The maximum absorption of 3:5-diiodotyrosine was (Table 1) at 2910 Å. in acid solution, with a molecular extinction coefficient of $2{\cdot}31 \times 10^3$; thus substitution of the two ortho positions by iodine produces an increase in the extinction of the phenolic group, and also a shift in the maximum of about 160 Å. In alkaline solution the increase in absorption, as compared with that of phenol, is even more marked; two maxima were observed, one at 3160 Å. ($\epsilon = 5{\cdot}26 \times 10^3$) and the other at 3060 ($\epsilon = 5{\cdot}17 \times 10^3$). The values reported here for diiodo-

Table 1. *Molecular extinction coefficients of tyrosine, tryptophan and 3:5-diiodotyrosine* ($\epsilon \times 10^{-3}$)

Substance	λ (Å.) 2600	2650	2700	2750	2800	2850	2900	2950	3000
Tyrosine at *p*H 13	1·66	1·14	0·96	1·14	1·50	1·80	2·15	2·30	1·97
Tyrosine at *p*H 1	0·53	0·76	1·09	1·29	1·21	0·75	0·15	0·02	—
3:5-Diiodotyrosine at *p*H 13	—	—	—	—	—	1·65	2·21	2·91	3·88
3:5-Diiodotyrosine at *p*H 1	1·14	1·32	1·46	1·63	2·01	2·21	2·30	2·09	1·52
Tryptophan at *p*H 13	2·95	3·82	4·32	4·88	5·43	4·87	4·10	2·29	1·25
Tryptophan at *p*H 1	3·25	4·10	4·72	5·16	5·49	4·52	3·41	1·40	0·58

Substance	λ (Å.) 3050	3100	3150	3200	3250	3300	3350	3400
Tyrosine at *p*H 13	1·25	0·70	0·35	0·09	—	—	—	—
Tyrosine at *p*H 1	—	—	—	—	—	—	—	—
3:5-Diiodotyrosine at *p*H 13	5·12	4·69	5·18	4·37	3·41	2·37	1·30	0·34
3:5-Diiodotyrosine at *p*H 1	0·62	0·23	—	—	—	—	—	—
Tryptophan at *p*H 13	0·52	0·14	0·05	—	—	—	—	—
Tryptophan at *p*H 1	0·09	—	—	—	—	—	—	—

tyrosine differ slightly from those obtained by Ginsel [1939].

The phenoxide ion of tyrosine absorbs very strongly between 2900 and 3100 Å., while the phenolic form absorbs little or no light in this region (Table 1). We have therefore used the extinction at the three wave-lengths of 2950, 3000 and 3050 Å. as a measure of the concentration of the phenoxide ion. Table 2 shows that mixtures of the two forms behave in accordance with the expectation that the absorption spectra of the two molecular species are additive and independent of each other.

Table 2. *Spectroscopic estimation of the phenolic dissociation constant of tyrosine*

Partly neutralized solutions of tyrosine were made up by mixing solutions of tyrosine in 0·01 *N* HCl with appropriate glycine buffer mixtures, to a final ionic strength of 0·04, care being taken to avoid contamination with CO_2. The concentrations of the phenoxide ion at different *p*H values were calculated from the extinctions at three different wave-lengths, with the aid of the values given in Table 1.

		*p*H	9·50	9·72	9·98	10·15	10·33	10·50
$\epsilon \times 10^{-1}$	(*a*)	at 2950 Å.	50	69	106	127	150	170
		pK calc.	10·05	10·03	10·05	10·06	10·06	10·05
,,	(*b*)	at 3000 Å.	41	59	90	107	123	141
		pK calc.	10·07	10·07	10·02	10·05	10·08	10·06
,,	(*c*)	at 3050 Å.	28	38	58	72	81	93
		pK calc.	10·04	10·08	10·04	10·02	10·07	10·04

The values obtained for the dissociation constant of the phenolic group of tyrosine vary little for different wave-lengths and at different *p*H values. The mean value for the *pK* is 10·05; the dissociation exponent for zero ionic strength—pK_0—can be extrapolated with the aid of the simplified Debye equation: $pK_0 = pK + 0{\cdot}5\sqrt{\mu}$. A value of 10·15 is thus obtained. Hitchcock [1924], by a combination of solubility and hydrogen electrode measurements, obtained two *pK* values for the alkaline dissociation of tyrosine. A *pK* of 9·2 was assigned to the amino-group, whilst the higher *pK* of 10·1 was assumed to correspond with the dissociation of the phenolic group. This interpretation is supported by our spectroscopic measurements.

The dissociation constant of the phenolic group in 3:5-diiodotyrosine was measured in a similar manner. The extinctions of partly neutralized diiodotyrosine solutions at the wave-lengths 3300, 3250, 3200 and 3150 Å. were determined and the *pK* calculated. A mean value of 6·42 was obtained. This is in good agreement with the value of 6·48 obtained by Dalton, Kirk & Schmidt [1930].

The state of tyrosine in insulin

The applicability to proteins of the spectrophotometric method, thus established for tyrosine and diiodotyrosine, depends mainly on the validity of the assumptions indicated above. Can the absorption spectra of proteins quantitatively be interpreted in terms of those of their constituent amino-acids? It has been shown that the introduction of peptide linkages changes the absorption spectrum of tyrosine very little [Fruton & Lavin, 1939], and Holiday [1936] has been able to estimate spectroscopically the tyrosine and tryptophan contents of several proteins, with results which are in fair agreement with those by other methods. For other proteins it has been necessary to introduce a rather unsatisfactory correction for 'pigment', which has also been discussed by Morton [1942]. Moreover gelatin, which contains only traces of tyrosine and no tryptophan, as shown by chemical methods, exhibits a small but definite band at 2800 Å., which is shifted towards the red in alkaline solution [Anslow & Assar, 1940]; these authors ascribe this band, which they also find in egg albumin, to peptide bonds. This interpretation, however, appears to be based on insufficient evidence.

Insulin has an amino-acid composition which simplifies the interpretation of spectroscopic data. It contains 12·5 % of tyrosine [du Vigneaud, 1938] and no tryptophan; 12·5 % of cystine, which is all present in the disulphide form; and some phenylalanine. The ultra-violet absorption spectrum of insulin has been examined by several authors, and was found to resemble that of tyrosine [Kuhn, Eyer & Freudenberg, 1931; Coulter, Stone & Kabat, 1936; Graubner, 1938].

Tables 3–5 and Fig. 1 give the experimentally determined molecular extinction coefficients of insulin at different *p*H values. With a molecular weight of 46,000 [Miller & Anderson, 1942], insulin contains 32 tyrosine and 24 cystine groups. The absorption of cystine in the relevant part of the spectrum is quite small; in order to calculate the tyrosine groups we have corrected, however, for cystine, using the values given by Ley & Arends [1932]. In acid solution (Table 3 and Fig. 2) the absorption of insulin at the maximum, i.e. between 2700 and 2800 Å., agrees quite well with the calculated value. At shorter wave-lengths the absorption can obviously not be explained by tyrosine alone; phenylalanine may be largely responsible for this excessive absorption. At the longer wave-lengths, i.e. at 2850 Å. and above, however, the absorption

of insulin is considerably greater than the calculated value, which is based on the assumption that tyrosine in insulin has the same absorption as the free amino-acid. The curve is very steep in this region and a slight shift of the absorption band of tyrosine incorporated in protein would greatly change the extinction coefficients. A shift of the whole band by about 20 Å. appears quite possible, and would account very satisfactorily for the whole absorption curve of insulin. It can be concluded that in acid solution the absorption of insulin above 2700 Å. is quantitatively explained in terms of that of tyrosine and that of cystine bound in disulphide linkage, and it appears unnecessary to postulate the presence of other absorbing groups, or of any linkage of the phenolic group of tyrosine.

Absorption of insulin in alkaline solution

In alkaline solution there is an increase of absorption below 2650 Å., which is probably not connected with the ionization of the phenolic group, since it occurs at *p*H 9. The absorption in the region from 2650 to 2800 Å. does not change appreciably if the *p*H is increased to 13. A further increase of alkalinity, however, is associated with a greatly increased absorption between 2650 and 2850 Å., and the maximum at 2950 Å., which is mainly due to the phenoxide ion, becomes less distinct. This 'unspecific' absorption which is connected with an unknown change in the molecule appears to spread to longer wave-lengths and at *p*H 13·8 the extinc-

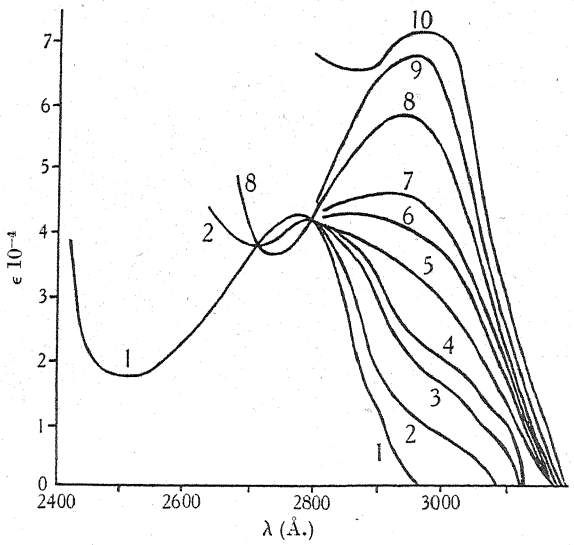

Fig. 1.

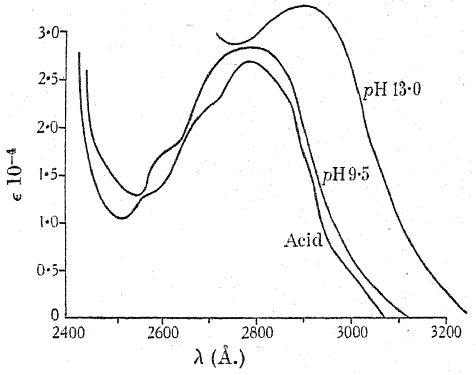

Fig. 2.

Fig. 1. Absorption spectrum of insulin. Curve no. 1 was obtained at *p*H 1, the figures 2–9 correspond to the *p*H values in Table 5, whilst curve 10 was obtained at *p*H 13·4.

Fig. 2. Absorption spectrum of egg albumin.

Table 3. *Molecular extinction coefficients of insulin at* p*H 1, with the number of tyrosine groups thus calculated*

λ (Å.) ...	2450	2500	2550	2600	2650	2700	2750	2800	2850	2900
$\epsilon \times 10^{-4}$	2·36	1·95	2·08	2·46	3·07	3·89	4·50	4·22	3·02	1·05
No. of tyrosine groups calculated	—	—	—	35·6	34·1	32·0	32·2	32·4	37·6	60·6

Table 4. *Molecular extinction coefficients of insulin at alkaline* p*H, with the number of tyrosine groups thus calculated*

		λ (Å.) ...	2750	2800	2850	2900	2950	3000	3050	3100	3150
$\epsilon \times 10^{-4}$	(*a*)	at *p*H 13·0	3·72	5·05	5·90	6·90	7·30	6·70	4·30	2·69	1·81
		No. of tyrosine groups calculated	29·1	31·5	31·2	31·2	30·4	33·5	34·0	36·4	50·4
,,	(*b*)	at *p*H 13·4	—	—	6·85	7·40	7·60	6·98	4·78	—	—
		No. of tyrosine groups calculated	—	—	36·1	33·1	32·0	35·0	37·5	—	—
,,	(*c*)	at *p*H 13·8	—	—	7·75	7·89	7·76	7·41	5·21	—	—
		No. of tyrosine groups calculated	—	—	41·1	34·9	33·6	37·0	41·0	—	—

tion coefficients at 3000 and 3050 Å. substantially exceed the calculated value.

It will be shown later that the phenolic groups in insulin dissociate at a higher *p*H than do those in tyrosine, and the ionization will therefore not be quite complete at *p*H 13·0. It can be calculated that approximately 30 groups out of 32 are dissociated at that *p*H. The absorption at *p*H 13·0 is fully explained if we assume again the slight shift postulated above. At higher alkalinity the values found are much too high, which must be due to 'unspecific' absorption. Generally speaking, however, it can be concluded that the absorption curve of insulin between the limits of *p*H 1 and 13 can be completely accounted for by its amino-acid composition, if we assume a slight shift of the curve for tyrosine incorporated in protein, in comparison with that for the free amino-acid.

Change of absorption of insulin with change in p*H*

Having thus established a quantitative correlation between absorption spectrum and stoichiometric composition, we studied the change of the spectrum as a function of the *p*H in greater detail (Table 5 and Fig. 1). The desired *p*H was obtained by mixing CO_2-free NaOH with a portion of a concentrated solution of insulin made up in 0·01*N* HCl, and diluting to the required volume; the ionic strength in most instances was less than 0·01. The time interval between the addition of alkali, the completion of the spectroscopic measurements, and the estimation of *p*H, was less than 10 min. The calculation of the *pK* was based on the assumption that 94% of the tyrosine groups in insulin are ionized at *p*H 13, which is equivalent to an effective *pK* of 11·8 over the very alkaline range. It is further assumed that the differences between the absorption spectra at *p*H 1 and 13, within the limits of 2950 and 3050Å., are entirely due to tyrosine; the shift in the tyrosine spectrum is thus taken into account. The *pK* values calculated from the data of Table 1 are not very different, but the agreement at the same *p*H and different wave-lengths is less satisfactory. A slight error in the first assumption will not significantly affect the results.

It can be seen from Table 5 that agreement at any one *p*H for different wave-lengths is satisfactory, but that the apparent *pK* increases considerably as the *p*H rises. The mean *pK* found for the phenolic group in insulin is about 11, as compared with 10·1 for that of tyrosine. The *pK* is calculated as if insulin were a monobasic acid so far as tyrosine is concerned; such a method of calculation would be correct if the tyrosine groups were placed so far from each other, and from any other group which could influence the intrinsic *pK* of the phenolic group, as to render such an influence negligible. This is obviously not the case. We may safely assume that the effect of charged groups will be the most important factor; substitution by polar but uncharged groups will not affect the *pK* greatly, unless such substitution were to take place in the phenolic ring itself. Calculated for a molecular weight of 46,000, insulin has 8 arginine, 32 histidine, 4 lysine and 24 free α-amino-groups [Chibnall, 1942]. The number of free carboxyl groups is not known with any accuracy, but we deduce from the titration data of Harington & Neuberger [1936] that there are approximately 50 such groups. At *p*H 9·9 all the iminazole and most of the α-amino-groups will be ionized, while all the guanidino and most of ε-amino-groups will still retain their positive charges. The total net charge of the insulin molecule at that *p*H will be approximately −40. At *p*H 12·3 most of the tyrosine groups will have dissociated, most of the lysine groups become unionized, and the net charge increased to about −70. The increase in apparent *pK* of the phenolic group runs parallel with this increase of net charge of the molecule, and it is concluded that the shift of the apparent *pK* of tyrosine in the protein, as compared with that of free tyrosine, is largely accounted for by the increase in the negative charge of the protein as the *p*H is raised. On this 'net

Table 5. *Molecular extinction coefficients of insulin at different* p*H values, with the* pK *values for the phenolic groups thus calculated*

*p*H values at which determinations were made ...	9·9	10·2	10·45	10·8	11·35	11·8	12·3	13·0
(*a*) $\epsilon \times 10^{-4}$ at 2950 Å.	1·46	2·27	2·76	3·88	4·56	5·04	6·44	7·30
No. of phenolic groups ionized	5·4	8·6	10·6	15·5	18·4	20·4	26·3	30·0
pK calc.	10·63	10·63	10·75	10·83	11·25	11·55	11·64	11·82
(*b*) $\epsilon \times 10^{-4}$ at 3000 Å.	1·12	1·87	2·34	3·39	4·11	4·53	5·87	6·7
No. of phenolic groups ionized	4·6	8·0	10·2	15·4	18·2	20·1	26·3	30·0
pK calc.	10·67	10·68	10·78	10·83	11·23	11·57	11·65	11·82
(*c*) $\epsilon \times 10^{-4}$ at 3050 Å.	6·7	1·21	1·76	2·47	2·94	3·25	3·96	4·30
No. of phenolic groups ionized	4·4	8·1	12·4	17·0	20·3	22·5	27·5	30·0
pK calc.	10·69	10·67	10·65	10·75	11·12	11·43	11·52	11·82
$pH_b - pH_s$	0·55	0·55	0·65	0·7	1·1	1·4	1·5	1·7

electrostatic effect' there may be superimposed a specific local effect of some substituents, say of neighbouring guanidino groups, on particular tyrosine groups, but such specific local effects are probably of minor importance compared with that of the total net charge. It has so far been assumed that the charge distribution on the surface of the protein molecule is symmetrical; there is no definite evidence that this is so in the case of insulin. The low dipole moments found for many proteins [Oncley, 1942] seem to indicate, however, that proteins in general do not show any marked asymmetry of charge distribution and, in the absence of any evidence to the contrary, this generalization is considered to apply to insulin too.

Instead of considering the *p*H as constant throughout the solution, and treating the *pK* of the phenolic group as a variable dependent upon the net charge, we might equally well, and perhaps more usefully, consider the intrinsic ionization tendency of the ionizing group as constant, and the *p*H on the surface of the protein—pH_s—as variable and depending on the bulk *p*H—pH_b—and on the net charge [Danielli, 1937; 1941; Hartley & Roe, 1940]. Because of its negative charge, the surface of the protein at an alkaline *p*H will contain more protons than the bulk of the solution, and the ultra-violet 'colour' of the phenolic groups will serve as an indicator of pH_s. It must be remembered, however, that the *p*H of the surface as defined by our measurements is not quite the same as the pH_s as used by Danielli [1941] in his study on the 'protein error' of indicators. In the latter case the *p*H of a volume element of the solvent adjoining the surface is considered, while we estimate the *p*H at a point on the actual surface of the molecule itself. The differences between bulk *p*H and surface *p*H, which are numerically identical with the differences between the *pK* of the phenolic group in the protein, and that of free tyrosine, are recorded in Table 5; the values are not strictly accurate, since no correction for the variation of ionic strength was made, but they give an indication of the magnitude of the shift of *pK* with increasing net charge, according to the first view, or of the difference between pH_b and pH_s according to the Hartley-Danielli definition.

If the generalization be accepted that the surface of protein molecules is electrically uniform, then the change of the dissociation constants of ionizable groups in the protein is entirely dependent on the size, shape and net charge of the molecule. Spherical molecules of the same molecular-weight class, like egg albumin and insulin, should therefore show an identical behaviour if the net charge is the same. It can also be predicted that in the neighbourhood of the I.E.P. the dissociation constants will be approximately the same as those found in the simple amino-acids or peptides, whilst in very acid media, or in most cases even more in alkaline solution, a considerable shift is to be expected.

Most of the spectroscopic data refer to a molecule outside the stability range of insulin, as defined by the sedimentation method [Miller & Anderson, 1942]. The changes occurring at moderate alkalinity are, however, reversible [Sjögren & Svedberg, 1931] and we believe that our data, which were obtained at *p*H values of 12 or less, represent characteristics of the unchanged, or reversibly-changed, insulin molecule.

Absorption spectrum and composition of egg albumin

Egg albumin presents a much more complicated picture. In acid solution there is a point of maximum absorption at about 2800 Å.; at *p*H 13 there is very marked increase of absorption between 2950 and 3150 Å. and the maximum is shifted to about 2900 Å. (Fig. 2). Qualitatively this behaviour is to be expected for a molecule containing both tyrosine and tryptophan. The quantitative analysis of the spectrum in terms of these two amino-acids is, however, not quite satisfactory.

There is a certain amount of disagreement about the molecular weight of egg albumin; we have accepted Chibnall's [1942] figure of 43,000 which is in fair agreement with most recent data. We have also used his data for tyrosine and tryptophan, i.e. we assume that egg albumin contains 10 tyrosine and 3 tryptophan groups per molecule. Table 6 gives the molecular extinction coefficients found at different *p*H values, and also the calculated extinction coefficients. The latter data were obtained by use of the figures for extinction coefficients given in Table 1, with the assumption that no phenolic groups are dissociated at *p*H 9·5 and that all 10 groups are ionized at *p*H 13.

Table 6. *Molecular extinction coefficients of egg albumin*

λ (Å.) ...		2500	2550	2600	2650	2700	2750	2800	2850	2900	2950	3000	3050	3100
$\epsilon \times 10^{-4}$														
(*a*) at *p*H 4·9	Found	1·12	1·13	1·45	1·81	2·26	2·51	2·85	2·55	1·90	0·86	0·48	—	—
	Calc.	—	—	1·50	1·99	2·50	2·84	2·86	2·11	1·17	0·44	0·18	—	—
(*b*) at *p*H 9·5	Found	1·48	1·27	1·70	1·90	2·54	2·83	2·83	2·66	2·00	1·16	0·68	0·35	0·19
	Calc.	—	—	1·42	1·91	2·39	2·84	2·84	2·21	1·38	0·71	0·38	0·16	0·04
(*c*) at *p*H 13·0	Found	—	—	—	—	2·65	3·28	3·28	3·42	3·50	3·28	2·73	1·51	0·95
	Calc.	—	—	—	—	2·25	3·13	3·13	3·26	3·38	3·04	2·34	1·41	0·74

At *p*H 4·9 the agreement between calculated and found values is quite satisfactory at the maximum, i.e. at 2800 Å.; at the shorter wave-lengths the experimental values are too low by about 10%, while at 2850 Å. and higher the extinction coefficients found are much larger than those calculated. These very high values at longer wave-lengths are also observed at *p*H 9·5, and to a lesser extent at *p*H 13. This discrepancy, which is here more marked than is the case with insulin, may be due to a number of causes. The absorption bands of tyrosine and tryptophan present in the protein may differ from those of the free amino-acids. Such a shift would be expected if these groups were linked to other groups by hydrogen bonds [Morton & Stubbs, 1940]. An examination of the fine structure of the tryptophan band in the egg albumin spectrum has revealed such a shift towards longer wave-lengths [Holiday, 1942]. It is also possible that another group is responsible for the increased absorption, as has been suggested by Anslow & Assar [1940]. A third possibility is that some of the increased 'absorption', particularly that above 3200 Å., is actually due to scattering of light, but an extrapolation to shorter wave-lengths with Rayleigh's formula, which was attempted by Custers, de Boer & Dippel [1933] for gelatin, appears unsatisfactory, since the refractive index of the protein for the light which is absorbed by the molecule, will probably be different from that found for light to which the protein is transparent. It seems unlikely that the discrepancy found can be explained by scattering. If allowance is made for the shift of absorption bands in the protein, the absorption spectrum of egg albumin between *p*H 1 and 13 can be almost quantitatively explained in terms of tyrosine and tryptophan, so that the change observed between *p*H 9·5 and 13·0 must be mainly due to the ionization of the phenolic group. An exact quantitative estimation on the basis of the present data is, however, somewhat uncertain.

Change of absorption spectrum of egg albumin on denaturation

If the *p*H of an egg albumin solution is increased from 9·5 to 12 little change in the absorption spectrum occurs, but if the *p*H is raised to 13 the spectrum changes very markedly (Table 6 and Fig. 2). If a solution exposed to *p*H 13 be brought back to *p*H 12 the absorption between 2900 and 3150 Å. is very much larger than that of a solution of *p*H 12 and the same ionic strength not previously exposed to *p*H 13 (Fig. 3). Exposure to *p*H 13 also denatured the protein, and it appeared likely that the irreversible change of the absorption spectrum, and the denaturation, were interrelated phenomena. This was shown to be the case. Denaturation by acid, urea, alkali and heat all produced the marked increase of absorption at *p*H 12, for wave-lengths above 2850 Å. (Table 7). Denaturation was demonstrated by the appearance of a precipitate on adjusting the *p*H to about 5, and also by a positive nitroprusside reaction. Exposure to *p*H 12 for 72 hr. at room temperature produced no denaturation and no change of absorption

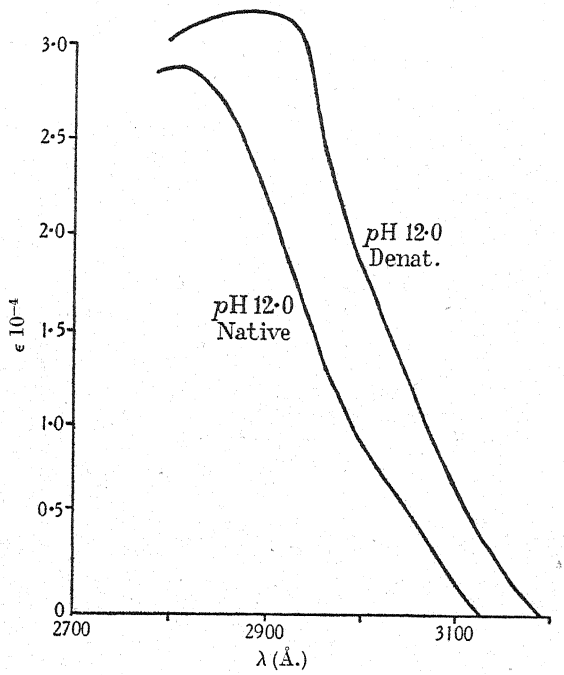

Fig. 3. Absorption spectrum of native and denatured egg albumin at *p*H 12.

spectrum. Denaturation at *p*H 13, however, appeared to be instantaneous. This is in agreement with the findings of Cannan [1942], who noted that the speed of denaturation of egg albumin by alkali is proportional to the fourth power of the hydroxyl ion concentration.

The extinction coefficients of preparations of the same protein which have been denatured by different means are approximately the same, and the increase in absorption must be connected with the mechanism of denaturation. The lack of ionization of the phenolic groups in the native protein, demonstrated here spectroscopically, is also confirmed by the titration data of Cannan, Kibrick & Palmer [1941]. The absorption spectrum indicates that on denaturation the phenolic groups are able to ionize at a *p*H lower than 12. This interpretation is supported by the change of absorption of the denatured protein with *p*H. Our data do not permit of an accurate estimation of the dissociation constant of the phenolic group in the denatured protein; the *pK* appears, however, to lie between 11·4

Table 7. *Absorption spectra of native and of denatured egg albumin*

The stock egg albumin solution contained 7·59 g. of protein/100 ml. of solution. For urea denaturation 1·89 g. of urea was added to 2 ml. stock solution, and the mixture was allowed to stand for 45 min. Acid denaturation was produced by adding 3 ml. 0·1 *N* HCl to 1 ml. of stock solution and keeping the mixture at 35° for 6 hr. Heat-denatured protein was obtained by adjusting the protein solution first to *p*H 7·5, and then heating it to 85° for 8 min. At that *p*H the solution remained clear. After dilution of these solutions, their *p*H was suitably adjusted by the addition of acid or alkali.

Egg-albumin solution	λ (Å.)	$\epsilon \times 10^{-4}$
(*a*) Urea-denatured at *p*H 10·75	2950	1·51
	3000	0·96
	3050	0·55
(*b*) Urea-denatured at *p*H 11·55	2950	2·01
	3000	1·53
	3050	0·96
(*c*) Urea-denatured at *p*H 12·0	2950	2·78
	3000	1·89
	3050	1·25
(*d*) Alkali-denatured at *p*H 12·0	2950	2·75
	3000	1·84
	3050	1·21
(*e*) Acid-denatured at *p*H 12·0	2950	2·70
	3000	1·78
	3050	1·18
(*f*) Heat-denatured at *p*H 11·3	2950	1·60
	3000	0·97
	3050	0·60
(*g*) Heat-denatured at *p*H 12·0	2950	2·60
	3000	1·71
	3050	1·18
(*h*) Native at *p*H 12·0	2950	1·49
	3000	0·95
	3050	0·50

and 11·8, a value not very different from that found for insulin. The difference in extinction coefficients between native and denatured protein at *p*H 12 corresponds to the ionization of 5–6 tyrosine groups, and from the probable value of the *pK* it must be concluded that at least 7–8 phenolic groups are concerned. It is possible that about 2 phenolic groups can ionize in the native protein, and this may account for the slight increase of absorption observed between *p*H 9 and 12; but this is uncertain.

The interpretation of the spectroscopic changes in terms of the acquired ionization of the phenolic groups on denaturation appears well founded, but the cause of this is, at present, a matter for speculation. It may be that phenolic groups in the native protein have *pK* values larger than 12·5, due to the specific configuration of the molecule, and that on denaturation the unfolding of the protein causes a change in *pK*. Such an assumption implies a highly asymmetrical charge distribution in the native protein which is very unlikely. It is also possible that the tyrosine groups are not on the surface of the molecule and are thus not able to react with hydroxyl ions. This interpretation also seems improbable; it is unlikely that the interior of the molecule is inaccessible to small ions like OH^-. Furthermore this theory would claim a position for tyrosine groups different from that of all other ionizable groups, which can react normally with H^+ and OH^-. It seems most probable that the phenolic OH groups are bound, in the native protein, in a linkage which is broken by denaturation. This interpretation is supported by the behaviour of native and denatured egg albumin to reagents such as ferricyanide [Mirsky & Anson, 1936], Folin's phenol reagent [Herriott, 1938] and porphyrindine [Brand & Kassell, 1940].

Mirsky & Pauling [1936] have postulated hydrogen bonds in proteins and have suggested that the breaking of such bonds may be largely responsible for denaturation. Phenols are known to form comparatively strong H bonds, but most relevant observations have been made either in non-polar solvents or on crystals. One of the few examples of H bond formation in water of phenols with groups other than those of the solvent molecules is that with salicylic acid or the salicylate ion, and a similar type of bond between the phenolic group of tyrosine and a carboxyl group of another amino-acid residue may exist in native proteins. The negative charge of the COO^- ion would stabilize the bond appreciably, and the bond would definitely increase the acid strength of this COOH, as in the case of salicylic acid [Branch & Yabroff, 1934]. SH groups may also be masked by the formation of weaker hydrogen bonds [Hopkins & Hunter, 1942]. Moreover, unknown groups may be concerned in a similar bond formation. The large number of such bonds, of which those formed by tyrosine will probably be the strongest and therefore the most important, would endow the native configuration of the protein with a certain degree of stability.* Strongly alkaline solution will cause ionization of the phenolic groups and thus break the hydrogen bonds; in order to effect denaturation it may be necessary to break several of these bonds simultaneously and thus the speed of denaturation will increase with a high power of the OH^- concentration [Cannan, 1942]. The mechanism of denaturation by heat at neutral *p*H may be very similar, and may also involve ionization of the phenolic hydroxyl group. Denaturation by acid may be due to a different mechanism. Addition of H^+ to COO^- will transform a strong H bond, stabilized by the negative charge, into a weaker bond, although no definite figures can be given for the relative bond energies in the acid and in the ion. Such a picture

* Brand & Kassell [1942] have very recently suggested a similar theory for the denaturation of lactoglobulin.

of denaturation, in which the H bonds formed by the phenolic group are largely, but not exclusively, responsible for the configuration of the native protein, is in accordance with other known facts. If a protein is irradiated with ultra-violet light it becomes either denatured, or changed in such a way that it becomes insoluble on a very slight increase of temperature [Clark, 1935]. It has also been found by Svedberg & Brohult [1938] that haemocyanin is split by irradiation with light of $\lambda = 2800$ Å. If hydrogen bonds formed by phenolic groups are present in these native proteins, then these effects of irradiation with light which is absorbed by the tyrosine grouping, are not surprising. Insulin cannot be denatured according to the usual meaning of the word; its tyrosine groups also appear to be free and able to ionize. We may assume that the large number of —S—S— bonds present in the molecule are largely responsible for the stability of this protein.

SUMMARY

1. The change of the absorption spectrum with *p*H has been used to estimate the dissociation constants of the phenolic groups of tyrosine and of 3:5-diiodotyrosine. pK values of 10·15 and 6·4 respectively were obtained, which are in good agreement with those obtained by other methods.

2. The absorption spectrum of insulin can be accounted for by the tyrosine and cystine present in the molecule if a slight shift of the tyrosine band in the protein be assumed. The change of the insulin spectrum with *p*H indicates that the pK of the phenolic group increases as the *p*H is raised. This change in the dissociation constant is assumed to be due mainly to the increasing net charge of the protein.

3. The absorption spectrum of egg albumin cannot be quantitatively interpreted in terms of its content of tyrosine and of tryptophan. The changes of absorption with *p*H, however, clearly indicate that in the native protein few or none of the phenolic groups are able to ionize, whilst after denaturation the occurrence of ionization can be demonstrated spectroscopically. The bearing of these results on the mechanism of denaturation is discussed.

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The Excretion of Phosphatase in the Faeces

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The mucous membrane of the small intestine is rich in phosphatase which can be liberated into the blood stream or into the intestinal secretion. This accounts for a part of the faecal phosphatase. Estimation of the faecal phosphatase may therefore enable one to study biochemically the activity of the mucous membrane of the small intestine in the intact animal.

The early publications on faecal phosphatase are reviewed by Folley & Kay [1936]. Heymann [1933] found the phosphatase activity to be reduced in rickets, spasmophilia and intestinal infantilism. Armstrong [1936] described the preparation of a strong phosphatase concentrate from dog faeces. Koster's [1938, 1939] most striking finding was the absence of phosphatase from the faeces in some cases of non-tropical sprue. In leukaemia values above the normal range were found. Duckworth & Godden [1941] used faecal phosphatase estimations to measure intestinal secretory activity in rats.

The experiments to be described were performed on the stools of normal adults between 20 and 35 years of age, free from any gross peculiarities of dietetic habit.

EXPERIMENTAL

Estimation of phosphatase

Technique. The stool was weighed, ground with water until homogeneous in a 1 in 400 suspension. Phosphatase was estimated by Bodansky's [1933] method. The increase in 1 hr. in inorganic phosphate, expressed as mg. P/100 g. of untreated or of dried faeces, is referred to as units of phosphatase activity (wet wt.) and (dry wt.) respectively. The former is comparable with the Bodansky unit of phosphatase activity.

Reliability of sampling. Subjects were instructed not to eat foods likely to leave large undigested particles. Grinding of their stools with water gave thorough mixing, and triplicate analyses showed that representative 0·5 g. samples could be obtained.

RESULTS

Solubility of the enzyme

Filtration of the suspensions removed a large and variable proportion of the phosphatase. Thus a stool which contained $2{\cdot}00 \times 10^3$ units (wet wt.) was tested again on four filtrates and the following results obtained: $0{\cdot}11 \times 10^3$, $0{\cdot}13 \times 10^3$, $0{\cdot}13 \times 10^3$, $0{\cdot}15 \times 10^3$ units. Substitution of saline for water did not affect the activity of the suspension.

Activation of the enzyme by Mg

$MgCl_2$ was added to give concentrations of 0·025 and 0·05 % (approx. 0·005 *M*) which was found by Bowers, Outhouse & Forbes [1940] to be optimal for dog faecal phosphatase. The phosphatase in human faeces is not fully activated with regard to Mg (Table 1), but any difference obtained by activation with Mg is negligible in comparison with the individual variations occurring physiologically.

Table 1. *Activation of faecal phosphatase by $MgCl_2$*

Phosphatase activity in units wet wt. $\times 10^{-3}$

Stool no.	Conc. added $MgCl_2$ in digest: Nil	0·025 %	0·050 %	Max. activation % increase
1	3·22	3·58	3·54	11
2	4·28	4·66	4·66	9
3	4·32	4·25	4·62	7
4	15·3	17·3	14·8	13
5	3·52	3·80	3·90	11
6	5·20	—	5·60	8
7	8·6	—	9·6	12
8	9·2	—	10·2	11
9	10·0	—	10·4	4
10	7·34	—	8·00	9

Inhibition of the enzyme by KCN

The results (Table 2) show that this phosphatase is strongly inhibited by 10^{-2} *M* KCN.

Table 2. *Inhibition of faecal phosphatase by KCN*

Stool	Sample	KCN conc. in digest *M*	Phosphatase activity units wet wt.	Inhibition %
A	1	Nil	$2{\cdot}64 \times 10^3$	95
	2	10^{-2}	$0{\cdot}13 \times 10^3$	
	3	Nil	$2{\cdot}74 \times 10^3$	96
	4	10^{-2}	$0{\cdot}11 \times 10^3$	
B	1	Nil	$2{\cdot}96 \times 10^3$	94
	2	10^{-2}	$0{\cdot}11 \times 10^3$	
	3	Nil	$3{\cdot}42 \times 10^3$	97
	4	10^{-2}	$0{\cdot}09 \times 10^3$	

Effect of NaF on activity of the enzyme

Experiments similar to those using KCN (Table 3) show that NaF has no effect upon the phosphatase activity of these preparations.

Table 3. *Effect of NaF upon faecal phosphatase activity*

Stool	Sample	NaF conc. in digest *M*	Phosphatase activity units wet wt.	Activation %
A	1	Nil	$4{\cdot}08 \times 10^3$	1
	2	10^{-2}	$4{\cdot}13 \times 10^3$	
	3	Nil	$4{\cdot}20 \times 10^3$	0
	4	10^{-2}	$4{\cdot}20 \times 10^3$	
B	1	Nil	$3{\cdot}76 \times 10^3$	3
	2	10^{-2}	$3{\cdot}89 \times 10^3$	
	3	Nil	$3{\cdot}75 \times 10^3$	7
	4	10^{-2}	$4{\cdot}02 \times 10^3$	

The phosphatase activity of normal stools

Stools from 50 normal adults were analysed for phosphatase activity and water content. The results are given in Table 4.

Table 4. *Phosphatase activity of normal stools*

	Min.	Max.	Mean of 50
Units wet wt. $\times 10^{-3}$	0·8	36·7	6·0
Units dry wt. $\times 10^{-3}$	1·9	103·0	23·0
Dry matter in stool (%)	15·0	47·0	30·0

DISCUSSION

Cloetens [1939 *a*, *b*; 1940] has presented evidence that the alkaline phosphatase of animal tissues consists of two types, 'alkaline phosphatase I' and 'alkaline phosphatase II'. This classification is based upon activation or inhibition by Mg ions, CN and F. The present results indicate that faecal phosphatase belongs to the subdivision type II of Cloetens, of the group A I of Folley & Kay [1936]. Cloetens found that nearly all the phosphatase of the small intestine of guinea-pigs was of this type.

The most striking feature of the analyses of normal stools is their extreme range of variation; but even the lowest value ($0{\cdot}8 \times 10^3$ units wet wt.) is 200 times the maximum value for normal serum. The values were very unevenly distributed: 36 of them (dry wt. basis) fell below the mean and only 14 above. The maximum value observed by Koster [1939] if converted into comparable units (dry wt.) is 94×10^3, which is close to the present maximum value of 103×10^3. If the mean phosphatase activity of the pooled faeces from six normal rats studied by Duckworth & Godden [1941] is converted into comparable units, a value of $1{\cdot}8 \times 10^3$ (wet wt.) is obtained, which lies within the range of $0{\cdot}8 \times 10^3$ to $36{\cdot}7 \times 10^3$ units (wet wt.) given above for normal human stools.

There is probably a negative correlation between the percentage of dry matter in normal stools and their phosphatase activity. Twenty-four stools containing less than 30 % of dry matter had an average of 33×10^3 units (dry wt.) and 26 stools containing 30 % or more dry matter had 14×10^3. The speed of transit of the stool through the intestine probably influences dry-matter content and phosphatase activity in opposite senses. Koster [1939] states that $MgSO_4$ taken by mouth, or a diet rich in green vegetables, increases the phosphatase activity of the stools. This effect he attributes to activation of the enzyme by Mg ions. Both procedures are, however, likely to increase the speed of transit of the intestinal contents and diminish the percentage of dry matter in the stools.

SUMMARY

1. The phosphatase activity of normal human faeces has been shown to be slightly increased *in vitro* by $0{\cdot}53 \times 10^{-2} M$ $MgCl_2$, uninfluenced by $10^{-2} M$ NaF, and very strongly inhibited by $10^{-2} M$ KCN.

2. The range of variation of the phosphatase activity of 50 normal stools was from $1{\cdot}9 \times 10^3$ to 103×10^3 units (dry wt.) with a mean value of $23{\cdot}0 \times 10^3$ units.

3. There is probably a negative correlation between the phosphatase activity/unit wt. dry matter and the percentage dry matter in normal stools.

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The Amino-acid Composition of Tyrocidine

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Tyrocidine hydrochloride, a crystalline antibacterial substance obtained, with gramicidin, from cultures of certain bacteria [Hotchkiss & Dubos, 1941] has been shown [Hotchkiss, 1941; Christensen, Edwards & Piersma, 1941] to be of polypeptide nature. A limited study of the constituent amino-acids has been made by these authors, and by Lipmann, Hotchkiss & Dubos [1941]. Dubos [1942] and Dubos & Hotchkiss [1942] have reviewed its biological properties.

We have studied the products of acid hydrolysis of tyrocidine by partition chromatography [Gordon, Martin & Synge, 1943 *a*, *b*]. The extraction of the acetylation mixture with organic solvents has been rendered almost quantitative, and the use of pelargonin chloride instead of methyl orange has practically eliminated 'leaching' of indicator, facilitating continuous observation of the more slowly moving 'bands'. These changes have extended the quantitative applicability of the method to acetamino-acids, whose bands move more slowly than that of acetylalanine, and although further investigation is necessary before the method can be used for the simultaneous determination of the eight common amino-acids of this group, the present procedure has proved useful with tyrocidine which seems to contain only three of these. Using a variety of solvent systems, we have not yet achieved a satisfactory chromatographic separation of the acetyl derivatives of glutamic and aspartic acid, and have made use of the differential evolution of CO_2 in the presence of ninhydrin from the corresponding free amino-acids [Van Slyke, Dillon, MacFadyen & Hamilton, 1941] for estimating their relative proportions.

From tyrocidine hydrolysates we have isolated and identified as their acetyl derivatives, phenylalanine, leucine, valine, proline, tyrosine, glutamic acid and ornithine, and have obtained further evidence for the presence of tryptophan and aspartic acid [Hotchkiss, 1941]. Alanine, glycine, lysine and hydroxyproline appear to be absent. Christensen *et al.* [1941] claim to have isolated alanine (as its dioxpyridate) but give no details.

All these amino-acids have been obtained predominantly as the *l*-isomers, except phenylalanine, which we find predominantly *d*-, and tryptophan, whose optical configuration was not determined. Many of the specimens isolated were considerably racemized. Our estimate of the total amount of *d*-amino-acids in the hydrolysate is consistent with that of Lipmann *et al.* [1941]. This seems to be the first report of *d*-phenylalanine in nature. The simultaneous incorporation by the same organism of *d*-leucine residues (in gramicidin) and *l*-leucine residues (in tyrocidine) has biological interest.

Ornithine was isolated as its di-acetyl derivative which proved identical with that obtained from *l*-ornithine, described here for the first time. Ornithine has previously been isolated only from hydrolysates of protein or polypeptide material that had been treated in such a way as probably to modify the guanidino group of arginine residues [Kossel & Weiss, 1913]. The negative Sakaguchi reaction of tyrothricin [Hotchkiss & Dubos, 1941] excludes arginine as an immediate precursor, although it must be borne in mind that tyrothricin is obtained from autolysed cultures. The presence of both proline and ornithine in the hydrolysate suggested that citrulline might be a common precursor for both [Wada, 1933], particularly since tyrocidine, like most proteins, gives a positive Fearon [1939] reaction. However, *l*-citrulline, when subjected to the same conditions of acid hydrolysis, acetylation and chromatographic analysis, gave at most 8 % of a mol. of proline and 22 % of a mol. of ornithine. Since under the same conditions of acid hydrolysis citrulline evolved no CO_2, while urea, another possible product of the reaction postulated by Wada, evolved 42–53 % of a mol. of CO_2, and also in view of his inadequate characterization of his product, we disbelieve Wada's statement that proline is the main product formed by boiling citrulline with acid. We believe that ornithine and proline residues exist in the intact molecule of tyrocidine, although the presence of some citrulline is not excluded. Further work on the Fearon reaction of proteins is desirable.

The satisfactory results with the estimation of ornithine given below confirm our view [Gordon *et al.*, 1943 *a*] that partition chromatography should provide a satisfactory micro-analytical procedure for the direct determination of lysine.

Quantitative analyses of tyrocidine hydrolysates were carried out and controlled by parallel analyses of a known mixture of the constituent amino-acids in similar quantity and proportion (Table 1). We were struck by the very low and variable recovery

of tryptophan, as in similar control experiments with a different amino-acid mixture [Gordon *et al.*, 1943*b*] higher and less variable recoveries of tryptophan were obtained. Homer [1915] and Onslow [1921, 1924] suggested that other amino-acids might some component not present in our first mixture. Gortner and collaborators (for references cf. Gortner & Norris [1923]) studied the role of tryptophan in humin formation. In our experiments humin formation is negligible although destruction of

Table 1. *Analysis of a known mixture which had been heated with acid under the conditions of hydrolysis employed with tyrocidine*

Known mixture		Analytical results (recovery %)	
Substance present	Amount mg. N	Exp. 1 (batch 'E' gel)	Exp. 2 (batch 'F' gel)
Acetyl-*l*-phenylalanine	0·348	106	101
Acetyl-*d*-leucine	0·290	98·5	105
Acetyl-*l*-valine	0·248	102	102
Acetyl-*l*-tryptophan	0·430	7	42
Acetyl-*l*-proline hydrate	0·158	91	93
Acetyl-*l*-tyrosine	0·167	99	104
Total dicarboxylic acids	0·343	79·5	96
l-Glutamic acid HCl	0·172	45	98 (by diff.)
l-Aspartic acid	0·171	113	94 (by ninhydrin)
Diacetyl-*l*-ornithine	0·493	93·5	96
Ammonium sulphate	0·349	—	—

Table 2. *Analysis of a glutamic acid-tryptophan mixture which had been heated with acid in the presence of, or in the absence of, air*

Known mixture			Analytical results		
			Amino-acids		
Substance present	Amount mg.	Air present during heating ml. at N.T.P.	Found mg.	Recovery %	Carbon dioxide formed mg.
l-Glutamic acid HCl	2·9	0·6	2·46	84	0·14
Acetyl-*l*-tryptophan	6·7		4·05	60	
l-Glutamic acid HCl	2·9	0·0	2·67	92	0·00
Acetyl-*l*-tryptophan	6·7		4·69	70	

Table 3. *Amino-acid analysis of tyrocidine*

(N as % of total N unless otherwise stated)

	Present results				
Preparation analysed ...	A I	A II	B —	Hotchkiss [1941]	Christensen Edwards & Piersma [1941]
mg. N taken for analysis ...	1·56	3·12	2·47		
Phenylalanine	12·2	14·8	12·7	—	12·9
Leucine	8·6	7·4	8·7	—	—
Valine	7·1	8·0	6·5	—	—
Tryptophan	9·5	0·9	1·8	11·7, 15·9*	4·7–6·8
Proline	6·4	6·6	7·1	—	—
Tyrosine	6·4	6·0	6·8	—	7·1
Total dicarboxylic acids	—	12·2	14·1	16·5	15
Glutamic acid (by diff.)	—	6·1	7·2	—	—
Aspartic acid (ninhydrin)	—	6·1	6·9	4·5	—
Ornithine	—	9·3	13·2	—	—
Ammonia	—	—	—	12·0*, 13·3	12·8
Batch of gel used in analysis	E	E	F		

* Determined after brief hydrolysis.

increase the destruction of tryptophan in hot acid, while Holm & Gortner [1920] studied the destruction of tryptophan heated alone with acid. All these workers allowed free access of air during hydrolysis, which is known to influence the destruction of tryptophan. In our experiments air was excluded, and we provisionally attribute the increased destruction to the interaction of tryptophan with tryptophan is almost complete. If this loss is due to interaction with other amino-acids, some destruction of these might be expected, and the results in Table 1 suggest that proline or glutamic acid might be involved. Preliminary experiments described below with tryptophan-glutamic acid mixtures were not decisive, although the evolution of CO_2 when air was present (Table 2) is of interest,

since proteins as usually hydrolysed yield significant quantities of CO_2 [Dunn, 1925]. The hydrolysis of tyrocidine, with exclusion of air, in our hands yielded no CO_2.

The accuracy of the analyses for the other amino-acids appears similar to that previously observed [Gordon *et al.*, 1943*a*]. Some of the minor differences between analyses 1 and 2 (Table 1) are undoubtedly due to differences in the adsorptive properties of the two batches of silica gel employed.

On applying the same analytical procedure to tyrocidine hydrolysates (cf. Table 3) we obtained very variable figures, the extent of the fluctuations being much greater than the experimental error of the method as established by the control experiments. Destruction of tryptophan was with one exception practically complete. We conclude that there are real differences in the composition of different hydrolysates. Incomplete hydrolysis seems an unlikely explanation, since bands corresponding with acetylpeptides should have been seen [Gordon *et al.*, 1943*c*], whereas the appearance of the chromatograms in the analysis of tyrocidine was in no way qualitatively different from that in the control experiments, exactly corresponding 'artefact bands' being observed. Diketopiperazines, however, if present, would not have been observed. We tentatively attribute the discrepancies to the different course which the interaction of tryptophan and other amino-acids may take under the conditions of hydrolysis, when these substances are present in the form of polypeptides rather than as free amino-acids or their simple derivatives.

Further research is required to elucidate the behaviour of tryptophan during acid hydrolysis of proteins. If the existence of interaction with other amino-acids or NH_3 is established, this introduces yet a fresh uncertainty into the deduction of the amino-acid composition of intact molecules from the composition of hydrolysates as usually prepared. With the average protein, of low tryptophan content, the percentage errors introduced in this way would not be as great as with tyrocidine. Nevertheless, they might invalidate stoichiometric calculations for a large molecule.

Whatever general significance for amino-acid analysis our observations may have, the errors encountered have entirely prevented the stoichiometric calculation of a 'minimum molecule' of tyrocidine hydrochloride, since molecules of a composition within the accuracy of our determinations can easily be postulated having two or any larger number of Cl′ groups, while we cannot rigidly exclude a molecule having only one Cl′ group.

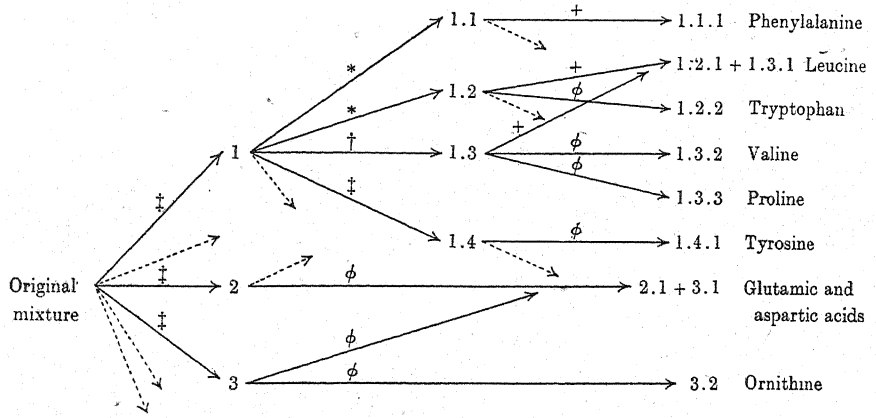
Nevertheless, if we combine Hotchkiss's tryptophan and NH_3 figures with those now obtained for other constituents, we can say that 88–105 % of the N of tyrocidine has been identified in its hydrolysate. A reliable estimate of the molecular weight by physical means might throw considerable further light on the data presented here.

EXPERIMENTAL

General analytical procedure
[cf. Gordon *et al.*, 1943 *a*, *b*]

Unless otherwise stated, preparations were hydrolysed by heating for 24 hr., at the temperature of boiling 6*N* HCl, with 0·8 ml. acetic acid and 3·0 ml. 6*N* HCl in sealed tubes which had been freed from air by boiling the mixture *in vacuo* before sealing [Gordon *et al.*, 1943*b*].

The acetylation and extraction procedure has been modified as follows: 5 ml. 4*N* NaOH were used in place of 10 ml. 2*N* NaOH. After acidification the solution was immediately absorbed in 16 g. silica gel. After thorough



* Fraction eluted with 1% butanol-$CHCl_3$
† Fraction eluted with 3% butanol-$CHCl_3$
‡ Fraction eluted with 17% butanol-$CHCl_3$
\+ Fraction eluted with 5% propanol-cyclohexane
ϕ Fraction eluted with 30% propanol-cyclohexane

The dotted lines in the diagram correspond to the 'artefact bands' observed during the fractionation.

mixing this was made to a slurry with 17% butanol-$CHCl_3$ (half-saturated with water), and made into a column in the usual way. No indicator was used. The vessels were washed through three times with small quantities of solvent, which were then added separately to the top of the column and allowed to drain in. Finally, fresh solvent to a total of 1 l. was run through the column. This multi-plate 'chromatographic' extraction utilizes solvent much more efficiently than the single-plate separating funnel procedure previously employed.

The scheme of fractionation shown above was employed. The initial fractionation with 17% butanol-$CHCl_3$ was introduced to ensure complete solution of the material for analysis, which does not occur when 1% butanol-$CHCl_3$ is used first. The silica was saturated with a 0·05% aqueous solution of pelargonin chloride [Gordon *et al.*, 1943*a*].

In calculating N values from the titrations, the following factors were used: ornithine and tryptophan, 2 atoms N; glutamic and aspartic acids, $\frac{1}{2}$ atom N; other fractions 1 atom N per acid equivalent.

Determination of glutamic and aspartic acids

After titration, fraction (2·1+3·1) was hydrolysed with 6*N* HCl for 6 hr. at 100°, evaporated to dryness *in vacuo* and dissolved in water. A suitable portion was taken for ninhydrin-CO_2 determination by the method of Christensen, West & Dimick [1941] with 10 min. heating. Calibration experiments showed that under these conditions glutamic acid evolved 1·02 mol. CO_2, and aspartic acid 1·88 mol. CO_2, and the relative amounts of the two amino-acids present were calculated from this.

CO_2 evolved during hydrolyses

Before analysis of their contents the hydrolysis tubes were connected (still sealed) to the Van Slyke-Neill manometric apparatus through pressure tubing; the tip of the tube was then broken off inside the rubber, the CO_2 transferred to the chamber and determined as described by Van Slyke *et al.* [1941]. Control experiments showed that no CO_2 was evolved by the HCl-acetic acid mixture during 24 hr. heating.

Preparation of tyrocidine hydrochloride

The material studied was obtained from the residues of our preparation of gramicidin from tyrothricin [Gordon *et al.*, 1943*b*]. Preparation A was obtained by three recrystallizations according to Hotchkiss & Dubos [1941]. After the 1st recrystallization, the material had $(\alpha)_D^{17\cdot5-18\cdot5°} = -98°$, after the 2nd, −100°, and after the 3rd, −100·5° (95% ethanol, $c = 1\cdot1$–$1\cdot3$). Prep. A had N = 14·5% (Kjeldahl, with or without P-HI pretreatment). Prep. B was obtained from A by one further recrystallization.

Characterization of tyrocidine constituents

244 mg. tyrocidine HCl (Prep. A) was hydrolysed and prepared for chromatography as described above, using double the quantity of each reagent. After making the hydrolysate alkaline, NH_3 was removed *in vacuo* before addition of acetic anhydride. The chromatographic fractionation was as above, with columns of suitably enlarged diameter. Pelargonin chloride was used as indicator throughout. Significant quantities of this were eluted by 30% propanol-cyclohexane though not by 17% butanol-$CHCl_3$. In these cases the contaminated fraction, dissolved in a little 17% butanol-$CHCl_3$, was filtered through a layer of 2 g. silica gel saturated with water. By washing with a suitable volume of fresh solvent the acetamino-acid was eluted free from pelargonin.

Acid equivalent weight (A.E.W.) and N (Kjeldahl) determinations were carried out as before [Gordon *et al.*, 1943*b*]; in the former, the first faint persistent pink of phenolphthalein was the end-point. C and H determinations were made by Dr G. Weiler, Oxford. Melting-points are given uncorrected unless otherwise stated. Optical rotations were observed in a 2 dm. tube.

*1.1.1. Acetyl-*d*-phenylalanine.* Crystallization from $CHCl_3$ gave a product (M.P. 160°) which on recrystallization yielded 39 mg. having M.P. 164°, which was depressed to 150–158° on admixture of authentic acetyl-*l*-phenylalanine (M.P. 169–171°). $(\alpha)_D^{19\cdot5°} - 37\cdot8°$ (ethanol, $c = 1\cdot1$). Du Vigneaud & Meyer [1932] record for acetyl-*d*-phenylalanine M.P. 172° (corr.), $(\alpha)_D^{26°} - 51°$ (ethanol). (Found: C, 63·6; H, 6·48; N, 6·53%; A.E.W. 211. Calc. for $C_{11}H_{13}O_3N$: C, 63·7; H, 6·28; N, 6·77%; A.E.W. 207.)

*1.2.1+1.3.1. Acetyl-*l*-leucine.* Crystallization from $CHCl_3$ yielded 17 mg. having M.P. 180–181°, not depressed on admixture of authentic acetyl-*l*-leucine. Admixture of acetyl-*d*-leucine depressed the M.P. to 160°. $(\alpha)_D^{20·5°} - 19·0°$ (ethanol, $c = 0·5$). Martin & Synge [1941] record for acetyl-*l*-leucine M.P. 182°, $(\alpha)_D^{24°} - 23·5°$ (ethanol, $c = 3·3$). (Found: C, 55·7; H, 8·93; N, 7·9%; A.E.W. 175. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%; A.E.W. 173.)

1.2.2. Acetyltryptophan would be expected in this fraction [Gordon *et al.*, 1943*b*]. Crystallization from $CHCl_3$-cyclohexane followed by recrystallization from $CHCl_3$ yielded 4·7 mg. having M.P. 170–190°, not depressed on admixture of acetyl-*l*-tryptophan. $(\alpha)_D^{20·5°}$ slightly positive ($< +3°$) (ethanol, $c = 0·15$). After evaporation from ethanol solution N content was 9·1% (P-HI pretreatment), and A.E.W. = 205. (Calc. for $C_{13}H_{14}O_3N_2$: N, 11·4%; A.E.W. 246.) Perhaps this fraction was a mixture of acetylleucine and acetyltryptophan. In two of the three quantitative analyses described below this fraction was also very small; in the other a substantial acid fraction was found.

*1.3.2. Acetyl-*l*-valine.* Crystallization from $CHCl_3$ yielded 13 mg. having M.P. 158°, not depressed on admixture of authentic acetyl-*l*-valine. The crystals were contaminated by a water-insoluble gummy material, possibly derived from tryptophan, which proved difficult to eliminate. $(\alpha)_D^{19°} + 2 \pm 1°$ (ethanol, $c = 0·35$). Gordon *et al.* [1943*a*] record for acetyl-*l*-valine M.P. 164°, $(\alpha)_D^{19°} + 4·0°$ (ethanol, $c = 2$). (Found: C, 54·8; H, 8·22; N, 8·0%; A.E.W. 176. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8%; A.E.W. 159.)

*1.3.3. Acetyl-*l*-proline (monohydrate).* Crystallization from wet ethylacetate yielded 23 mg. having (air-dry) M.P. 76–78°, not depressed on admixture of authentic acetyl-*l*-proline hydrate. $(\alpha)_D^{19°} - 112·5°$ (H_2O, $c = 0·7$) (rotation calc. for anhydrous compound). Martin & Synge [1941] record M.P. 78°, $(\alpha)_D^{22°} - 114·0°$ (H_2O, $c = 4·2$). Analyses were on the air-dry hydrate. (Found: C, 47·2; H, 7·26; N, 7·7%; A.E.W. 184. Calc. for $C_7H_{11}O_3N.H_2O$: C, 48·0; H, 7·43; N, 8·0%; A.E.W. 175.)

*1.4.1. Acetyl-*l*-tyrosine.* Treatment with ethanol-light petroleum eliminated much gummy material, after which

were slowly deposited 17 mg. crystals (slightly contaminated by gum) having M.P. 148°, not depressed on admixture of authentic acetyl-*l*-tyrosine. $(\alpha)_D^{19.5°}+41°$ (H_2O, $c=0.4$). Du Vigneaud & Meyer [1932] record M.P. 152–154° (corr.), $(\alpha)_D^{26°}+47.5°$ (H_2O, $c=2$). (Found: C, 57·6; H, 5·53; N, 5·9%; A.E.W. 252. Calc. for $C_{11}H_{13}O_4N$: C, 59·3; H, 5·83; N, 6·3%; A.E.W. 223.)

2.1+3.1. Dicarboxylic amino-acids. A preliminary determination on a portion of this fraction (*R* (chromatogram band rate) = 0·2 (approx.) on both 17% butanol-$CHCl_3$ and 30% propanol-cyclohexane columns [Gordon *et al.*, 1943*a*]) showed exactly 2 acid equiv./atom N, suggesting the presence of dicarboxylic acids only. Since attempts at crystallization led only to mixed products, the material was refractionated on a 17% butanol-$CHCl_3$ chromatogram, and the front and back portions of the resulting broad band were collected separately. Crystallization of the former material from ethanol-$CHCl_3$ yielded 6 mg. having M.P. 190°, not depressed on admixture of authentic *acetyl-l-glutamic acid.* Bergmann & Zervas [1928] record M.P. 199° (corr.). (Found: C, 44·9; H, 5·71; N, 8·4%; A.E.W. 91. Calc. for $C_7H_{11}O_5N$: C, 44·4; H, 5·82; N, 7·4%; A.E.W. 94·5.) The material available did not suffice for optimum analytical accuracy.

In a second analysis, the corresponding fraction from the hydrolysate of 210 mg. of Prep. A was hydrolysed (6 hr. in 6*N* HCl at 100°), and evaporated to small volume. The dicarboxylic acids were separated in the usual way. Saturation with HCl gas at 0° gave slow deposition of glutamic acid HCl crystals (16 mg.), having $(\alpha)_D^{18.3°}+13°$ (H_2O, $c=0.3$) (rotation calc. for glutamic acid). Wood [1914] records for *l*-glutamic acid in water + 1 mol. HCl $(\alpha)_D^{25°}+27.9°$. (Found: N, 7·3%; A.E.W. 93. Calc. for $C_5H_9O_4N$.HCl: N, 7·6%; A.E.W. 92.)

The mother liquors from the above, after most of the HCl had been removed *in vacuo*, were treated with copper acetate in 2 ml. water. The crystalline Cu salt deposited was filtered off and decomposed in the usual way with H_2S, yielding material (5·2 mg.) which did not readily crystallize from water, having N content (7%) lower than the theoretical for aspartic acid. After treatment with excess HCl followed by evaporation to dryness, the material had $(\alpha)_D^{18°}+13°$ (H_2O, $c=0.2$) (rotation calc. for dry wt. before HCl treatment). Wood [1914] records for *l*-aspartic acid in H_2O + 0·94 mol. HCl $(\alpha)_D^{25°}+18.4°$. A ninhydrin-CO_2 determination showed the same CO_2/N ratio as aspartic acid (see above). From this identity, the insolubility of the Cu salt, the —COOH/N ratio, and the fact that the rate and appearance of the dicarboxylic acid chromatogram band from tyrocidine hydrolysates could be closely simulated by mixtures of acetylglutamic and acetylaspartic acids, we conclude that the fraction under consideration was an impure specimen of aspartic acid, and that tyrocidine hydrolysates contain *l*-glutamic and *l*-aspartic acids, the former considerably racemized.

3.2. NN′-diacetyl-l-ornithine. Crystallization from ethylacetate and from ethanol-ether successively yielded 25 mg. needles having M.P. 156°, not depressed on admixture of authentic diacetyl-*l*-ornithine (see below). $(\alpha)_D^{16°}+4°$ (ethanol, $c=0.4$). (Found: C, 50·6; H, 7·77; N, 12·3%; A.E.W. 224.) On 17% butanol-$CHCl_3$ and 30% propanol-cyclohexane chromatograms the band-rate *R* was approx. 0·1, and the material was chromatographically indistinguishable from authentic diacetyl-*l*-ornithine on a butanol-$CHCl_3$ column, while readily separable from diacetyl-*dl*-lysine [cf. Gordon *et al.*, 1943*a*].

Acetylation of l-*ornithine*

l-Ornithine HCl prepared from *l*-arginine HCl by the action of liver arginase [Hunter, 1939] and having $(\alpha)_D^{18°}+10.8°$ (H_2O, $c=5$) was subjected to acetylation and extraction as described above, and yielded material similar in appearance and crystallization properties to the product from tyrocidine, having M.P. 156° (constant on recrystallization), $(\alpha)_D^{15°}+6.3°$ (ethanol, $c=0.8$). (Found: C, 49·9; H, 7·68; N, 12·7%; A.E.W. 218. $C_9H_{16}O_4N_2$ requires: C, 50·0; H, 7·40; N, 13·0%; A.E.W. 216.)

Quantitative analyses of tyrocidine hydrolysates, etc.

Three analyses were performed, according to the general scheme given above, on 10–20 mg. tyrocidine HCl with 3 g. silica gel in 1 cm. tubes for the butanol-$CHCl_3$, and 2 g. for the other fractionations. Methyl orange was substituted for pelargonin in those columns where the development was begun with 1% butanol-$CHCl_3$ or 5% propanol-cyclohexane, because of its superior sensitivity.

Control analyses on a known mixture after heating in acid are shown in Table 1. Similar experiments on simple mixtures of glutamic acid and acetyltryptophan were carried out in the presence and absence of air. In the former analysis air was readmitted to the 20 ml. tube to a pressure of 4 cm. Hg after the tube had been completely evacuated. The analytical procedure was simplified by omitting the propanol-cyclohexane chromatograms. Table 2 indicates the slightly higher recoveries found in the absence of air and gives the CO_2 evolution measured as described above. No CO_2 was evolved by tyrocidine or by the control mixture or by 4·05 and 6·1 mg. *l*-citrulline (Hofmann La Roche—kindly provided by Prof. H. A. Krebs) when heated in acid under the same oxygen-free conditions. 1·65 and 2·5 mg. of urea similarly treated yielded 0·565 and 0·98 mg. CO_2 respectively. After 4 mg. citrulline had been treated as above the resulting material was analysed in the same way as the glutamic acid-tryptophan mixtures, yielding at most 8% of a mol. of acids having $R>0.5$ in 17% butanol-$CHCl_3$ (which would include acetylproline), and 22% of a mol. of diacetylornithine. These substances were not identified; the figures are based on titration of bands having corresponding rates. (For recovery of proline and ornithine from known mixture cf. Table 1.)

Table 3 shows the results (uncorrected) of our analyses of tyrocidine together with the figures obtained by other workers.

SUMMARY

1. Technical modifications have extended quantitative analysis by partition chromatography to a further group of amino-acids.

2. From an acid hydrolysate of tyrocidine, phenylalanine, leucine, proline, valine, tyrosine, ornithine and glutamic acid have been isolated by partition chromatography, and characterized as their acetyl derivatives. Additional evidence has been obtained for the occurrence of tryptophan and

aspartic acid. This appears to be the first isolation of ornithine by acid hydrolysis of an intact protein or polypeptide.

3. The phenylalanine isolated had mainly the *d*- configuration; the other amino-acids were mainly *l*-.

4. 88–105 % of the N of tyrocidine has now been accounted for, but the variability of the analytical figures has not permitted stoichiometric calculations. The implications of this variability, which exceeds the analytical errors, are discussed.

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The Structure of the Galactose-phosphate Present in the Liver During Galactose Assimilation

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In this paper further support is provided for the view [Kosterlitz, 1937; 1938; 1939*b*] that in the galactose-phosphate present in the liver during galactose assimilation the phosphate group is attached to carbon atom 1. It has not yet been possible to separate this ester from the accompanying glycerophosphate, and from small quantities of glucose-1-phosphate and an unidentified ester. But the facts that reducing hexosemonophosphates can be removed by alkaline hydrolysis, and that Ba glycerophosphate is very resistant to acid hydrolysis and has a specific rotation of 0°, have made possible the approximate determination of the rate of acid hydrolysis of the galactose-phosphate and of its specific rotation. Since the ester is non-reducing, resistant to alkaline hydrolysis and very labile to acid hydrolysis, it is concluded that the first carbon atom is substituted. The velocity constant (k) of hydrolysis of the natural ester in $0{\cdot}25N$ HCl at 25° is $0{\cdot}91 \times 10^{-3}$, while that of synthetic galactose-1-phosphoric acid is $0{\cdot}89 \times 10^{-3}$. Natural Ba galactose-1-phosphate has $[\alpha]_{5461} + 113°$ while the synthetic ester has $[\alpha]_{5461} + 109{\cdot}5°$. These values strongly suggest that the natural and synthetic esters are identical. It is of interest to note that Wolfrom & Pletcher [1941] and Wolfrom, Pletcher & Brown [1942] have succeeded in finally proving that the glucose-1-phosphate of Cori, Colowick & Cori [1937] has the structure of phosphoryl α-mono-*d*-glucopyranoside. Since the principle of the synthesis of galactose-1-phosphate is analogous to that devised by Cori *et al.* [1937] for glucose-1-phosphate, the findings of Wolfrom *et al.* [1942] give further support to the view expressed on other grounds [Kosterlitz, 1939*a*] that the synthetic galactose-1-phosphate is phosphoryl α-mono-*d*-galactopyranoside.

EXPERIMENTAL

The general methods were the same as those described in previous papers [Kosterlitz, 1937; 1939*a*]. The Ba salts of the phosphoric esters were obtained

from the livers of 38 rabbits which had been fed with galactose. The method of isolation has been described previously [Kosterlitz, 1937]. No nitrogenous substances were present in the ester mixture. The yield of Ba salts was 1·75 g. from 2·41 kg. liver.

Analysis of Ba salts of the ester mixture. The substance contained 6·87% total P and 1·88% acid-labile P (=27·4% of the total P). The initial reduction was 9·1% 'glucose'; 12 min. hydrolysis in 0·09 *N* H_2SO_4 at 100° increased the reduction by 8·9% 'glucose' or 11·7% 'galactose'. The fermentation analysis gave the following results: change after fermentation with *Saccharomyces ludwigii* (*S. ludwigii*) = +2·8% 'glucose'; change after fermentation with galactose-adapted *Saccharomyces cerevisiae* (*S. cerevisiae*) Frohberg = −8·9% 'glucose'; residual reduction: 3·0% 'glucose'. The mixed Ba salts, therefore, contained 11·7% galactose as ester or 25·7% anhydrous Ba galactose-1-phosphate. Since Ba glycerophosphate was found to be more easily soluble in aqueous ethanol than Ba galactose-phosphate, a separation of the two compounds was attempted by fractional precipitation with aqueous ethanol. This attempt, however, was not very successful [Kosterlitz, 1938].

Acid and alkaline hydrolysis of the ester mixture. A sample of a composition slightly different from that described in the preceding paragraph was used for this particular experiment. 17·96 mg. Ba salt were dissolved in 15 ml. H_2O containing 0·87 ml. *N* H_2SO_4 and the precipitated $BaSO_4$ removed by centrifuging. The initial reduction was 7·76% 'glucose' and the total P 7·02%. The acid-labile P, determined by 3 min. hydrolysis in 0·09 *N* H_2SO_4 at 100°, was 1·84%; the alkali-labile P, determined by hydrolysis in 0·2 *N* NaOH at 100°, was 1·29% after 20 min. and 1·33% after 30 min. hydrolysis. The acid-labile P constituted 26·2% and the alkali-labile P 18·7% of the total P so that 55% of the total P was resistant to hydrolysis. Galactose-1-phosphate and glucose-1-phosphate were the main sources of the acid-labile P, and reducing hexosephosphates and possibly pentosephosphates those of the alkali-labile P.

Removal of reducing phosphate esters by hydrolysis in 0·2 N NaOH. 534 mg., containing 11·7% galactose (=62·5 mg.) as phosphate, were dissolved in 47·4 ml. H_2O and 2·58 ml. *N* H_2SO_4. The precipitated $BaSO_4$ was removed by centrifuging and washed with 5 ml. H_2O. To the total of 55 ml. were added 2·58 + 13·75 ml. *N* NaOH (final concentration = 0·193 *N*). The hydrolysis was allowed to proceed at 100° for 30 min., during which time the solution turned deeply yellow, due to the action of the alkali on the reducing sugars. After cooling and addition of 16·33 ml. *N* HCl, $Ba(OH)_2$ was added until the *p*H was 8·4. The precipitated Ba phosphate was centrifuged off and washed. To the supernatant fluid, ethanol was added to give a concentration of 60%. After 2 hr. standing at 0°, the material was centrifuged, the supernatant fluid decanted, and the precipitate successively extracted with 20, 20, 10, 10 and 15 ml. H_2O. The residue was dissolved in a minimum of 0·25 *N* HCl, the solution brought back to *p*H 8·4 by means of $Ba(OH)_2$, and then centrifuged. The supernatant fluid was added to the aqueous extracts, followed by ethanol to give a concentration of 60%. The extraction of the centrifuged precipitate was repeated twice and the final precipitate dried *in vacuo* over P_2O_5. Yield: 293 mg. This material together with 23 mg. from a previous experiment (containing 3·0 mg. galactose as phosphate) was dissolved in 26 ml. H_2O, the insoluble material separated and washed by centrifuging. To the clear solution (32 ml.) an equal quantity of ethanol was added; after standing at 0° for 2 hr., the Ba salts were centrifuged off, washed with 50 and 80% ethanol and dried *in vacuo* over $CaCl_2$. Yield: 295 mg. The slightly turbid solution of this product in 10 ml. H_2O was centrifuged, and an equal quantity of ethanol was added to the supernatant fluid. After standing at 0° for 2 hr., the precipitate was centrifuged off and then successively extracted with 15, 10 and 15 ml. 10% ethanol. Ethanol was added to the supernatant fluid to give a concentration of 50%. After 2 hr. standing at 0°, the precipitate was centrifuged off, washed with 80% ethanol and dried *in vacuo* over $CaCl_2$. The Ba salts were now completely soluble in H_2O. Yield: 228 mg. containing 16·3% = 37·2 mg. galactose as phosphate. The crude material contained 62·5 mg. (+3·0 mg.) galactose. Yield: 57%.

Analysis of the Ba salts of the ester mixture freed from reducing phosphates. The substance contained 7·46% total P and 2·77% acid-labile P (=37·2% of total P). $[\alpha]_{5461}^{20°}$ = +34·4 (*c* = 1·476; *l* = 2). The initial reduction was negligible (0·45% 'glucose') and 3 min. hydrolysis in 0·09 *N* H_2SO_4 at 100° increased the reduction by 12·4% 'glucose' or 16·3% 'galactose'. The fermentation analysis (Table 1) showed that most of this increase was due to galactose:

Table 1. *Analysis of saccharide phosphate mixture by fermentation*

	Unhydrolysed esters	Hydrolysed esters
Initial reduction	0·38% 'glucose'	12·9% 'glucose'
Change after fermentation with *S. ludwigii*	+10·9% 'glucose'*	− 1·53% 'glucose'
Change after fermentation with galactose-adapted *S. cerevisiae* Frohberg	−10·5% 'glucose'	−10·2% 'glucose'
	=13·8% 'galactose'	=13·3% 'galactose'
Residual reduction	0·72% 'glucose'	1·15% 'glucose'

* This increase in reduction was caused by phosphatatic hydrolysis of galactose-1-phosphate [Kosterlitz, 1937; 1939*a*].

From these results, the following conclusions may be drawn: (1) alkaline hydrolysis practically abolished the initial reduction which was caused by reducing hexosephosphates and possibly pentosephosphates; (2) the greatest part but not all of the reducing substances produced by acid hydrolysis consisted of galactose (13·5%). Besides galactose, 1·53% glucose was liberated, and 0·93% expressed as glucose of a non-fermentable reducing substance. Thus, the mixture of Ba salts contained as anhydrous salts: 29·6% Ba galactose-1-phosphate, 3·4% Ba glucose-1-phosphate, and 2·0% unidentified acid-labile ester (calculated on basis of hexose-phosphate). This made a total of 35% anhydrous Ba salt or 39·8% Ba salt containing $3H_2O$.

These results show up a fact which so far has escaped attention, namely, that during galactose assimilation, glucose-1-phosphate is present in the

liver besides galactose-1-phosphate. In experiments on rats, Kosterlitz & Ritchie [1943*b*] could demonstrate an increase in glucose-1-phosphate in the livers after administration of galactose as well as of glucose. Nothing can so far be said about the unknown acid-labile ester.

Hydrolysis in 0·09N H_2SO_4 at 100° of the ester mixture freed from reducing phosphates

(Concentration of acid-labile P = 0·525 m.-molar)

Time min.	Increase in reduction mg. 'glucose'/ mg. Ba salt	Increase in P mg./mg. Ba salt
2	0·125	0·0272
3	0·125	0·0277
10	0·126	0·0277
180	0·130	0·0318

The liberation of reducing substances and of P was practically complete after 2–3 min. hydrolysis; under the conditions of this experiment, galactose-1-phosphate is completely hydrolysed in 2 min. and glucose-1-phosphate in 5 min. [Kosterlitz & Ritchie, 1943*a*]. Between 10 and 180 min. there was an insignificant increase in reduction, while there was still a liberation of inorganic P; this would suggest the presence of a non-reducing difficultly hydrolysable ester (or esters) such as glycerophosphoric acid.

Isolation of the difficultly hydrolysable esters by acid hydrolysis of the ester mixture freed from reducing phosphates. 96·5 mg. Ba salts were dissolved in 29·5 ml. H_2O and 3·49 ml. *N* H_2SO_4 (= free esters in 0·09*N* H_2SO_4). This solution was hydrolysed at 100° for 10 min. After addition of $Ba(OH)_2$ (*p*H 8·4), the precipitate was centrifuged off and 2 vol. of ethanol added to the supernatant fluid. The precipitate was dissolved in the minimum amount of 0·25*N* HCl, the solution warmed in order to precipitate colloidal $BaSO_4$, and the $BaSO_4$ centrifuged off. $Ba(OH)_2$ was added to the supernatant fluid (*p*H 8·4), the precipitate of inorganic P centrifuged off, and 2 vol. of ethanol added. The $BaSO_4$ was warmed with 0·25*N* HCl and the HCl extract treated as described above. The combined aqueous alcoholic mixtures were allowed to stand for 2 hr. at 0°. After centrifuging, the precipitate was exhaustively extracted with H_2O and the centrifuged extracts treated with 2 vol. ethanol. After standing at 0° for 2 hr., the precipitate was centrifuged and the process of extraction repeated once with H_2O and a second time with 10% aqueous ethanol. The final precipitate was dried *in vacuo* over $CaCl_2$. Yield: 39 mg. Expected: 60% of 96·5 mg. = 58 mg.; yield: 67%.

Analysis of the Ba salt of the difficultly hydrolysable ester. Total P = 7·48%; inorganic P = 0; acid-labile P = 0. Initial reduction = 0·58% 'glucose'; acid-labile reduction = 0·3% 'glucose'. $[\alpha]_{5461}^{17.5°} = -3·4°$ ($c = 0·778$; $l = 2$).

Fermentation analysis: initial reduction = 0·61% 'glucose'; change after fermentation with *S. ludwigii* = +0·38% 'glucose'; change after fermentation with galactose-adapted *S. cerevisiae* = −0·57% 'glucose'; residual reduction = 0·42%.

This ester was practically non-reducing; it was not further analysed but was probably composed mainly of Ba α-glycerophosphate (+3H_2O = 8·6% P) which is present in liver in considerable quantities [Fiske & Subbarow, 1929]. However, the low P value and the slight laevo-rotation indicated that other unknown esters were also present.

Calculation of the specific rotation of natural Ba galactose-1-phosphate. The Ba salts of the ester mixture containing 29·6% anhydrous galactose-1-phosphate had $[\alpha]_{5461} + 34·4°$. By deducting −2·0° for the 60% believed to be present of the Ba salt of the difficultly hydrolysable ester, and +3·0° for 3·4% anhydrous Ba glucose-1-phosphate, a value of +33·4° was obtained, which corresponded to 29·6% anhydrous Ba galactose-1-phosphate. Thus, natural anhydrous Ba galactose-1-phosphate has approximately $[\alpha]_{5461} = +113°$, while for the anhydrous Ba salt of the synthetic ester $[\alpha]_{5461} = +109·5°$. No allowance could be made for the 2% unidentified acid-labile ester.

Hydrolysis of the acid-labile fraction of the ester mixture, freed from reducing phosphates in 0·25N HCl at 25°. 28·19 mg. Ba salt were dissolved in a total of 10 ml. with addition of 1 drop sat. K_2SO_4 solution and 1·365 ml. of 0·1*N* H_2SO_4, in order to liberate the free acid. After centrifuging off the $BaSO_4$, 9 ml. 0·5*N* HCl were added to 9 ml. of the supernatant fluid. The concentration of acid-labile P was 1·17 m.-molar.

Hydrolysis in 0·25N HCl at 25°

Time min.	Increase in reduction (% of total acid-labile reduction)	$k \times 10^3$	Inorganic P formed (% of total acid-labile P)	$k \times 10^3$
180	29·6	0·847	29·8	0·854
255	38·3	0·764	38·6	0·775
330	45·8	0·751	46·0	0·744
	Average	0·787		0·791

Calculation of the k of hydrolysis of natural galactose-1-phosphoric acid by 0·25N HCl at 25°. The acid-labile fraction of the ester mixture contained 84·6% galactose-1-phosphoric acid, 9·7% glucose-1-phosphoric acid, and 5·7% unidentified ester. The *k* of the acid-labile fraction was $0·79 \times 10^{-3}$. The *k* of glucose-1-phosphoric acid ($0·2 \times 10^{-3}$) was calculated from the known *k* values of the glucose-1-phosphoric and galactose-1-phosphoric acids at 25°, 37° and 50° [Cori *et al.*, 1937; Kosterlitz, 1939*a*; Kosterlitz & Ritchie, 1943*a*]. No allowance could be made for the 5·7% unidentified ester. By deducting $0·02 \times 10^{-3}$ for the 9·7% glucose-1-phosphate present in the acid-labile fractions a value of $0·77 \times 10^{-3}$ was obtained, which corresponded to 84·6% galactose-1-phosphoric acid. Thus, the *k* of natural galactose-1-phosphoric acid is approximately $0·91 \times 10^{-3}$, while the *k* of the synthetic ester is $0·89 \times 10^{-3}$.

SUMMARY

1. The mixture of phosphoric esters isolated from the livers of rabbits assimilating galactose was subjected to alkaline hydrolysis in order to remove reducing hexosephosphates.

2. The product obtained in this way contained 29·6% anhydrous Ba galactose-1-phosphate, 3·4% anhydrous Ba glucose-1-phosphate and 2% Ba salt of unidentified acid-labile ester; the remainder consisted of a difficultly hydrolysable non-reducing ester. The Ba salts of the ester mixture freed from

reducing phosphates had $[\alpha]_{5461} + 34{\cdot}4°$ and the Ba salt of the difficultly hydrolysable ester $[\alpha]_{5461} - 3{\cdot}4°$. From these values it was calculated that the natural anhydrous Ba galactose-1-phosphate has $[\alpha]_{5461} + 113°$, while the synthetic product has $[\alpha]_{5461} + 109{\cdot}5°$.

3. The velocity constant (k) of hydrolysis of the acid-labile fraction of the ester mixture, freed from reducing phosphates, in $0{\cdot}25N$ HCl at 25° was $0{\cdot}79 \times 10^{-3}$. From this value the k of the natural galactose-1-phosphoric acid was calculated to be $0{\cdot}91 \times 10^{-3}$, while the k of the synthetic product is $0{\cdot}89 \times 10^{-3}$.

4. These results strongly suggest that the natural and synthetic galactose-1-phosphoric acid are identical. The ester probably is an α-pyranoside as is the natural glucose-1-phosphoric acid.

An expenses grant by the Medical Research Council which partly defrayed the cost of this investigation is gratefully acknowledged.

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The Apparent Dissociation Constants of Galactose-1-Phosphoric Acid

By H. W. KOSTERLITZ, *From the Physiology Department, Marischal College, Aberdeen*

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Meyerhof & Suranyi [1926], Meyerhof & Lohmann [1927], and Cori, Colowick & Cori [1937] found that the dissociation constants (pK' values) of hexose-diphosphoric, hexose-6-monophosphoric, fructose-6-phosphoric, and glucose-1-phosphoric acids were smaller than those of orthophosphoric acid. In the present paper α(?)-galactose-1-phosphoric acid is shown to have dissociation constants similar to those of the other hexosemonophosphates. The knowledge of the acidic properties of galactose-1-phosphoric acid is necessary for the assessment of the changes in pH which may be brought about by the phosphorylation of galactose.

EXPERIMENTAL

The pH values were measured by means of a Cambridge pH meter with dip glass electrode (portable pattern). A buffer consisting of equal parts of Na acetate and acetic acid ($0{\cdot}1N$) and having pH 4·62 was used for standardizing the glass electrode. The galactose-1-phosphoric acid was prepared by treating the Ba salt (from the K_2 salt [Kosterlitz, 1939]) with the exact quantity of H_2SO_4. The concentration of galactose-1-phosphoric acid in the final solution was determined by estimation of phosphorus. 10 ml. of $0{\cdot}1015M$ (Exp. 1) and $0{\cdot}1038M$ (Exp. 2) galactose-1-phosphoric acid were titrated with CO_2-free $0{\cdot}0999N$ NaOH (temp. = 18–20°). For comparison, 10 ml. of $0{\cdot}1001M$ H_3PO_4 were similarly titrated. The results were plotted after correction for the deviations from 0·1 molarity

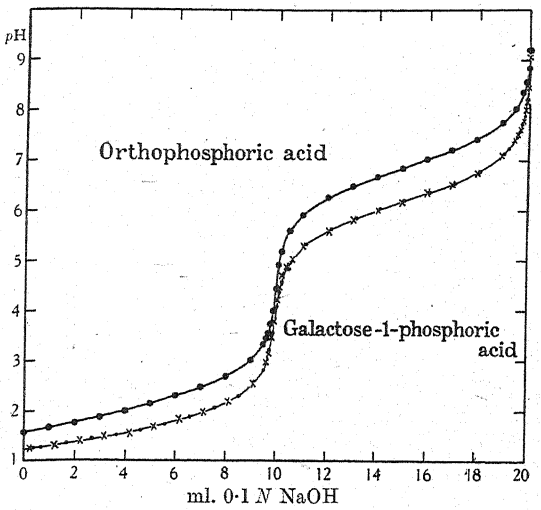

Fig. 1. 10 ml. $0{\cdot}1M$ orthophosphoric acid •—• 10 ml. $0{\cdot}1M$ galactose-1-phosphoric acid: Exp. 1 ·—·; Exp. 2. ×—×.

(Fig. 1). The dissociation constants were calculated from 3 to 4 values each; pK'_1 by means of

$$K'_a = \frac{[H]^+ (B + [H]^+)}{C - (B + [H]^+)}$$

and pK'_2 by means of Henderson-Hasselbalch's equation

$$pK'_a = pH - \log \frac{B}{C-B}$$

[Van Slyke, 1922]. In these equations B stands for molar concentration of total alkali added and C for molar concentration of galactose-1-phosphate in the titration mixture at any given time.

The values found for orthophosphoric acid, pK'_1 1·95 and pK'_2 6·83, agree well with those recorded in the literature [Van Slyke, 1922; Meyerhof & Suranyi, 1926; Cori *et al.*, 1937]. Galactose-1-phosphoric acid has pK'_1 1·00 and pK'_2 6·17 while hexose-6-phosphoric acid (Robison ester) has pK'_1 0·94 and pK'_2 6·11, fructose-6-phosphoric acid pK'_1 0·97 and pK'_2 6·11 [Meyerhof & Lohmann, 1927] and glucose-1-phosphoric acid pK'_1 1·10 and pK'_2 6·13 [Cori *et al.*, 1937]. There is no significant difference between the biologically important pK'_2 values of galactose-1-phosphoric acid and those of the other hexosemonophosphates.

Since Meyerhof & Lohmann [1927] found that the pK_2 values of fructose-6-phosphoric and hexose-6-phosphoric (Robison) acids were decreased by about 0·14 by the addition of 0·1 *M* neutral salt solution, 10 ml. 0·1 *M* K_2 galactose-1-phosphate solution were titrated with *N* HCl. pK'_2 determined in this way was 6·06, as compared with 6·17, the value obtained by titrating the ester with NaOH.

SUMMARY

α(?)-Galactose-1-phosphoric acid, like other hexose-monophosphates, is a stronger acid than is orthophosphoric acid. α(?)-Galactose-1-phosphoric acid has pK'_1 1·00 and pK'_2 6·17, while orthophosphoric acid has pK'_1 1·95 and pK'_2 6·83.

An expenses grant by the Medical Research Council which partly defrayed the cost of this investigation is gratefully acknowledged.

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The Fermentation of Galactose and Galactose-1-phosphate

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Galactose-1-phosphate is present in the livers of rabbits and rats during galactose assimilation [Kosterlitz, 1937; Kosterlitz & Ritchie, 1943*b*]. No information as to the fate of this ester has so far been obtained except that it is more resistant to the phosphatase of liver than is glucose-1-phosphate [Cori, Colowick & Cori, 1938; Kosterlitz & Ritchie, 1941]. Since the carbohydrate metabolism of yeast cells is strikingly similar to that of mammalian tissue, it was decided to examine the fermentation of galactose and galactose-1-phosphate and compare the results with those obtained in the fermentation of glucose and glucose-1-phosphate.

The ability of some yeasts to ferment galactose is due to the formation of 'galactozymase' during adaptation. This process takes place in aqueous solutions of galactose and can occur without cell multiplication [Stephenson & Yudkin, 1936; for the older literature see that paper, and Harden, 1932]. Yeast juice [Harden & Norris, 1910; Grant, 1935], dried yeast and maceration extract [Abderhalden, 1925] prepared from adapted yeast, will ferment galactose. In the products of fermentation Nilsson [1930] found, besides Harden-Young ester, a monophosphate with a specific rotation higher than that of Robison ester. No data as to the reducing power of this ester and the nature of its constituent hexose were given. Grant [1935] confirmed the presence of 1:6-fructose diphosphate, and found trehalose-monophosphate and Robison ester. Using the methylphenylhydrazine test, he could not find any evidence for the accumulation of a galactose phosphoric ester. He further could demonstrate that galactose-6-phosphate was not fermented by galactose-adapted yeast.

EXPERIMENTAL

Materials and methods

Maceration extracts. These were prepared in the usual manner from a pure strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) Frohberg, which had been propagated in this laboratory since 1935, when it was generously supplied by

Dr H. B. Hutchinson of the Distillers Company, Ltd. It has been subcultured in broth containing 0·8% dried 'Difco' yeast extract and 3% galactose. For the preparation of dried yeast, the yeast was grown in aerated broth in lots of 1½–3 l. at 25° for 48 hr.

Galactose. In order to remove glucose, a 10% solution of commercial galactose was incubated with 2% *Saccharomyces ludwigii* (*S. ludwigii*) at 28° for 48 hr.; after centrifuging off the yeast and concentrating the solution under reduced pressure, the galactose was crystallized several times from 70% ethanol. It had $[\alpha]_{5461}^{17°} + 95{\cdot}0°$.

Glucose-1-phosphate. The K_2-salt was prepared according to Hanes [1940].

Galactose-1-phosphate. The K_2-salt was prepared by the method given in a previous paper [Kosterlitz, 1939*a*]. Since the K_2-salts of the esters are alkaline, HCl was added to obtain the desired *p*H. The quantities of acid required were read from electrometric titration curves [Kosterlitz, 1943].

CO_2 production. This was followed in Warburg manometers shaken in a bath at 25°. The gaseous phase was N_2; any O_2 present in the commercial nitrogen was absorbed by passing through two gas-washing bottles containing a solution of 16 g. $Na_2S_2O_4.2H_2O$ and 2 g. Na-anthraquinone-2-sulphonate in 100 ml. 13% NaOH [Fieser, 1924] and through a third bottle containing 13% NaOH.

Estimation of glucose-1-phosphate and galactose-1-phosphate. The esters were determined in trichloroacetic acid filtrates by the method described by Kosterlitz & Ritchie [1943*a*].

RESULTS

The results of three typical experiments are given in Fig. 1*a–c*. In all experiments, whether with hexoses or esters, typical curves of CO_2 production were obtained, showing an induction period, a period of rapid fermentation (period of esterification), and finally, a period of slow fermentation (period of hexosediphosphate fermentation). Meyerhof & Lohmann [1927] found that only the naturally occurring hexosemonophosphates, namely, Neuberg and Robison esters, gave such curves, while synthetic esters which were not intermediaries in alcoholic fermentation produced CO_2 at a slow and uniform rate.

In the first experiment (Fig. 1*a*), notwithstanding the addition of a small quantity of hexosediphosphate to the fermentation mixture, the CO_2 curves of the free hexoses showed a fairly long induction period and an autocatalytic shape. The maximum rates of CO_2 production were 80 μl./5 min. with glucose, and 62 μl./5 min. with galactose. On the other hand, the curves obtained with glucose-1-phosphate and galactose-1-phosphate were practically identical. Their maximum rates were 45–50 μl. CO_2/5 min. It is to be noted that, since in these experiments inorganic P was present in excess, the shape of the CO_2 curves was wholly conditioned by the substrate. Thus, during the rapid period of fermentation, glucose produced 575 μl. CO_2 and galactose 500 μl. CO_2 instead of the expected 1090 μl., or about 50%, while the esters produced 300 μl. CO_2 or 27·5% of the theoretical value. This means that while the hexoses obeyed the Harden-Young equation the esters did not. These results are in good agreement with those of Meyerhof & Lohmann [1927] who, investigating the action of maceration extract on Neuberg and Robison esters, found a CO_2 production of 25% of the calculated value, and with those of Harden & Robison [Harden, 1932] who found that during fermentation with zymin, only 20–25% of the added Robison ester was fermented at the enhanced rate. The lower CO_2 production by the monophosphates can be explained by the facts that, compared with the hexoses, they can accept only one-half the quantity of phosphate and that, according to Warburg & Christian [1939], the partial dephosphorylation of 1:3-diphosphoglyceric acid to 3-phosphoglyceric acid by the hexokinase-adenosine diphosphate-hexose system is a limiting factor in alcoholic fermentation by maceration extract.

Addition of catalytic quantities of acetaldehyde and hexosediphosphate (Fig. 1*b*) almost abolished the induction period in the fermentation of the free hexoses, with a simultaneous increase of the maximum rates of CO_2 production. In the case of glucose the maximum rate rose from 80 to 160 μl./5 min., and in the case of galactose from 62 to 75 μl./5 min. Although the addition of acetaldehyde caused a marked difference between the fermentation rates of free glucose and free galactose, it scarcely influenced the fermentation of either of the 1-esters.

When acetaldehyde alone was added to the fermentation mixture, the difference between the fermentations of glucose and galactose became rather more marked than in the experiment with addition of acetaldehyde and hexosediphosphate. There was a slight difference between the rates of CO_2 production in the case of the 1-esters in favour of glucose-1-phosphate (Fig. 1*c*).

If the yeast was dried slowly in the manner suggested by Lipmann [1938], the maceration extract obtained from it was four times as active as were extracts from yeasts dried in the ordinary way. In the presence of acetaldehyde, the maximum rates of CO_2 evolution/5 min. were as follows: glucose 690 μl., galactose 270 μl., glucose-1-phosphate and galactose-1-phosphate 200 μl. With glucose-1-phosphate the maximum fermentation rate was attained sooner than with galactose-1-phosphate, otherwise there was no difference between the fermentations of the two esters.

The experiments described so far indicate that galactose-1-phosphate plays the same role in galactose fermentation as do glucose-1-phosphate and glucose-6-phosphate in glucose fermentation. Grant [1935] found that Robison ester and 1:6-fructose diphosphate accumulate during fermentation of galactose by adapted yeast preparations, and that

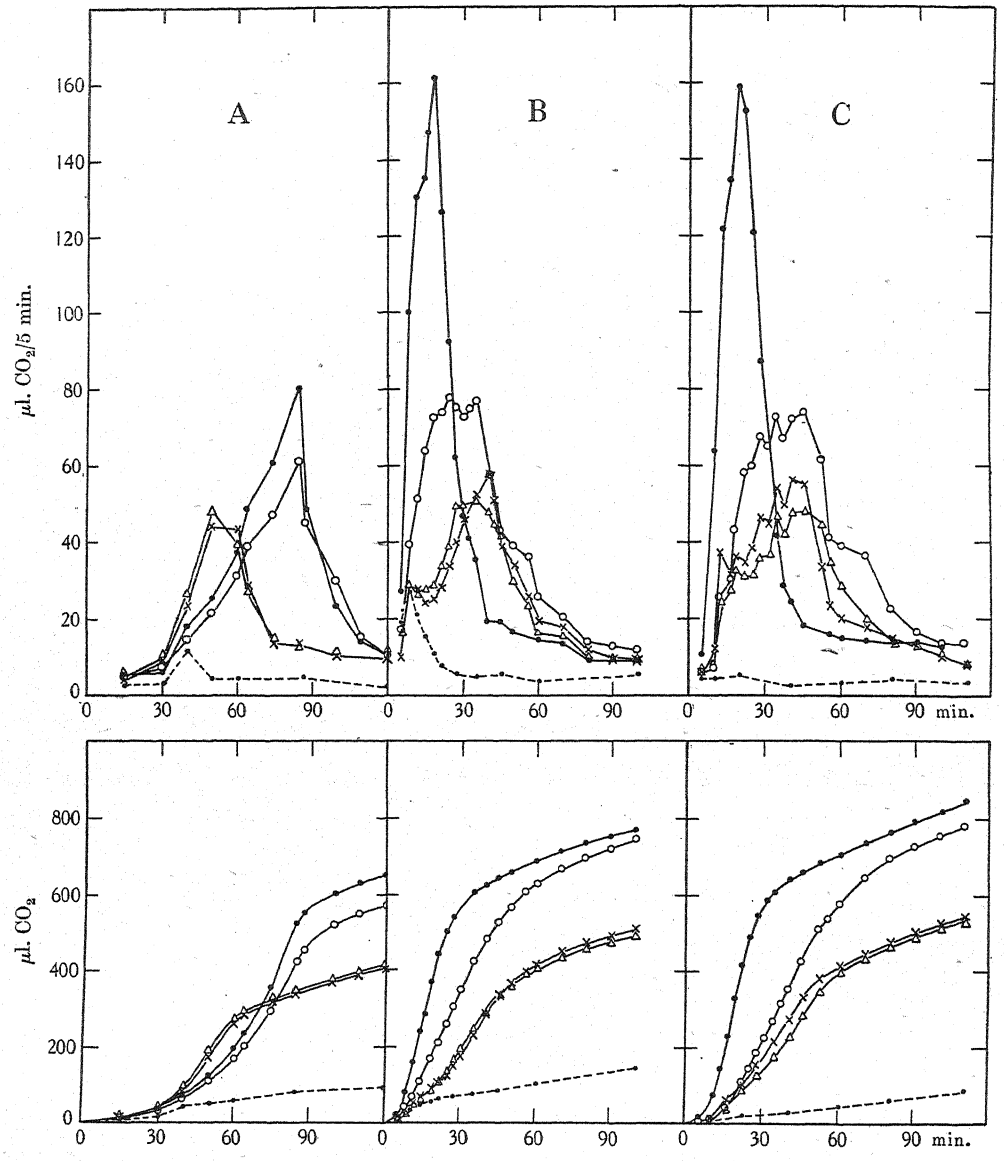

Fig. 1. Maceration extract from *Saccharomyces cerevisiae* Frohberg grown on galactose broth. 1 part dried yeast and 4 parts water were incubated at 35° for 2 hr. The extract was obtained by centrifuging at 4000 r.p.m. 1 ml. contained 2·55 mg. inorganic P; *p*H 5·8.

Main chamber: 1 ml. extract.

Side-bulb: (*a*) 0·05 ml. K_2-hexosediphosphate (0·115 mg. P), and either 0·25 ml. hexose solution (=4·37 mg. hexose=1090 μl. CO_2) in 0·097 *M* phosphate buffer of *p*H 6·5, or 0·25 ml. ester solution (=4·37 mg. hexose) in 0·0267 *N* HCl (*p*H 6·5).

(*b*) 0·05 ml. containing Mg-hexosediphosphate (0·07 mg. P) and 1 mg. acetaldehyde, and either 0·2 ml. hexose solution (=4·5 mg. hexose=1120 μl. CO_2) in 0·12 *M* phosphate buffer of *p*H 6·5, or 0·2 ml. ester solution (=4·5 mg. hexose) in 0·0338 *N* HCl (*p*H 6·5).

(*c*) as in (*b*), but hexosediphosphate was omitted.

····· Autofermentation; •—• glucose; o—o galactose; ×—× glucose-1-phosphate; △—△ galactose-1-phosphate.

galactose-6-phosphate is not an intermediary in galactose fermentation. The question, therefore, arises of how galactose-1-phosphate is converted to Robison ester: Is galactose-1-phosphate transformed to glucose-1-phosphate, which in turn is acted upon by phosphoglucomutase and isomerase to form Robison ester? Preliminary experiments with dialysed maceration extracts were not conclusive on account of the presence of phosphoglucomutase in such extracts. To give an example: 0·75 ml. extract, dialysed for 2 hr. + 0·5 ml. 0·08 *M* veronal acetate buffer of *p*H 6 + 0·25 ml. 0·06 *M* glucose-1-phosphate or galactose-1-phosphate adjusted to *p*H 6 were incubated at 25° for 30 min. In the case of glucose-1-phosphate 83 % of the ester was converted to difficultly hydrolysable ester (Robison), 14 % was accounted for by inorganic P, while 3 % remained unaltered. In the case of galactose-1-phosphate only 27 % was converted to difficultly hydrolysable ester, 11 % was accounted for by inorganic P, while 62 % remained unaltered. The structure of the difficultly hydrolysable ester formed from galactose-1-phosphate has not yet been identified but it is likely to be Robison ester, since galactose-6-phosphate can be ruled out. No glucose-1-phosphate was present in the reaction mixture of the experiment with galactose-1-phosphate, a finding which had to be expected in view of the presence of highly active phosphoglucomutase. Unfortunately, phosphoglucomutase cannot be inhibited specifically. Attempts to reduce the activity of phosphoglucomutase by prolonged dialysis were accompanied by great losses in the activity of the galactose-1-phosphate-converting enzyme; the activity could not be restored by Mg^{++} ions which, in extracts dialysed for 2 hr., accelerated the disappearance of galactose-1-phosphate. Fractionation with $(NH_4)_2SO_4$ has so far been unsuccessful; both the galactose-1-phosphate-converting enzyme and phosphoglucomutase were absent from the proteins precipitated by either 50 or 70 % saturation.

Notwithstanding the great differences between the fermentation rates of the non-phosphorylated hexoses, the fermentation curves of the 1-esters are so similar to each other as to suggest the existence of an enzymatic equilibrium between the esters. However, in order to obtain further information on the mechanism which causes the conversion of galactose-1-phosphate to Robison ester, the attempts to isolate the enzyme, or at least to separate it from phosphoglucomutase, will have to be taken up again on a scale larger than is possible at present.

From the facts described in this paper, a tentative hypothesis of galactose fermentation may be formulated as follows: During the adaptation of *S. cerevisiae* Frohberg to ferment galactose at least two new enzymes are formed. Enzyme (1) phosphorylates galactose at C_1, probably by a system similar to the hexokinase-adenylpyrophosphate systems which phosphorylate glucose and fructose at C_6 and not at C_1 [Colowick & Kalckar, 1941]. Enzyme (2) converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate, which in turn is acted upon by phosphoglucomutase or isomerase. The fermentation rate of galactose is lower than that of glucose; the rates of reaction (1) or (2) or of both constitute the limiting factors. During the process of adaptation, yeast may be fully adapted to ferment galactose-1-phosphate and, at the same time, be incompletely adapted to ferment non-phosphorylated galactose; when this is the case, galactose will be fermented more slowly than the 1-phosphate. This phenomenon, which was observed in two out of five samples of dried yeast [Kosterlitz, 1939*b*; 1942], supports the view that two new enzymes are formed during adaptation. Non-adapted yeast ferments neither galactose nor galactose-1-phosphate.

SUMMARY

1. With maceration extract, both glucose-1-phosphate and galactose-1-phosphate showed the characteristics of natural hexosemonophosphates, namely, the three periods of induction, rapid fermentation, and slow fermentation. While the non-phosphorylated hexoses produced about 50 % of the calculated quantity of CO_2 during the period of rapid fermentation, the esters produced only 25–30 % during the same period. The fermentation rates of the esters were so similar as to suggest an enzymatic equilibrium between them. So far it has not been possible to supply direct experimental proof for this suggestion.

2. A tentative hypothesis of galactose fermentation is formulated: it is assumed that during the adaptation of *Saccharomyces cerevisiae* Frohberg to ferment galactose, two new enzymes are formed, one which phosphorylates galactose at C_1 and another which converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate.

An expenses grant by the Medical Research Council which partly defrayed the cost of this investigation is gratefully acknowledged.

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The Oxidation of Ascorbic Acid by *o*-Dinitrobenzene, and the Detection of Dehydroascorbic Acid

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During a survey of the natural sources of ascorbic acid, three reactions have been observed that may be of analytical value: (1) the rapid reduction of *o*-dinitrobenzene by ascorbic acid to a substance giving a violet pigment in alkaline solution, (2) the formation of a green pigment when dehydroascorbic acid is boiled alone in aqueous solution at *p*H 3·5–4·5, and (3) the spontaneous formation of a red pigment when dehydroascorbic acid is kept in alkaline solutions out of contact with air.

(1) *Reaction of ascorbic acid with* o*-dinitrobenzene*

(*a*) *Experimental.* When 2–5 ml. of a dilute (0·02 %) aqueous solution of ascorbic acid are treated in the cold with 5 drops of a saturated aqueous solution of *o*-dinitrobenzene, and then made alkaline by addition of 5 drops 20 % NaOH, a violet colour rapidly develops, and is stable for several hours. Under these conditions the colour is not given by dehydroascorbic acid, cysteine, glutathione, uric acid or creatinine. Fructose gives a slow positive reaction in the cold, but only after 10–15 min., when the solution contains at least 0·2 % of this sugar. The other reducing sugars may require 2 hr. or longer. Proteins, simple alcohols, aldehydes, ketones, and the commoner hydroxy-, keto-, and amino-acids do not give the test. The test loses its selectivity if the reaction mixture is heated, and a positive result is then given by all the reducing sugars and by uric acid. Thus, by controlling temperature and concentration of alkali, it becomes possible to detect ascorbic acid in presence of other compounds of biological importance. On acidification, the violet pigment changes to yellow, and can be extracted by chloroform or by peroxide-free ether. Addition of alkali removes the pigment from the organic solvent, and regenerates the violet colour. By this means, small amounts of ascorbic acid can be detected in solutions the colour of which obscures the test.

(*b*) *History of the reagent.* Lipschitz [1920] reported that living tissues, such as muscle and yeast, contain a catalytic system capable of reducing '*m*-dinitrobenzene' to a yellow pigment, at *p*H 7·4, under anaerobic conditions. Addition of alkali converted the yellow into violet. The test was developed colorimetrically, and it was later found to depend on the presence of *o*-dinitrobenzene as a contaminant in the *m*-isomer [Lipschitz & Osterroth, 1924]. Apparently unaware of this, Ekkert [1934] claimed that reducing sugars gave a violet colour when heated in alkaline solution with *m*-dinitrobenzene, but v. Szecseny-Nagy [1935] showed that this reaction was due also to contamination by the *o*-isomer, and that pure *m*-dinitrobenzene gave no colour. This was confirmed by Truhaut [1937].

(*c*) *Mechanism of the test.* From an inspection of the formulae of the reactants, the test seems to depend on the presence of the enediol system, —C(OH)=C(OH)—, which occurs in ascorbic acid, though, of course, the reagent is not specific for this system, since powerful inorganic reducing agents such as hydrosulphite give a similar response.

Aldoses and ketoses react only when conditions bring about enolization. Kept in alkaline solution at room temperature for some hours they give an immediate violet on addition of the reagent, which

thus may be used to demonstrate the relative rate of transformation. Polyhydroxy compounds incapable of enolization, such as glycerol, mannitol, sorbitol, mucic and tartaric acids, do not react. Pyrogallol, when dissolved in oxygen-free water, and protected from atmospheric oxidation by a layer of toluene, gives an immediate reaction, as also does 1:3-dihydroxyacetone, and glucoreductone.

The pigment formed by the reduction of *o*-dinitrobenzene has been investigated by Bamberger & Hübner [1903], and by Meisenheimer [1903]. Using hydroxylamine as the reducing agent, the end-product was identified as *o*-nitro-nitrosobenzene, and isolated as the Na salt. Working under less drastic conditions, Patzig & Patzig [1906] were able to recover *o*-dinitrobenzene from the pigment after acidification and atmospheric oxidation.

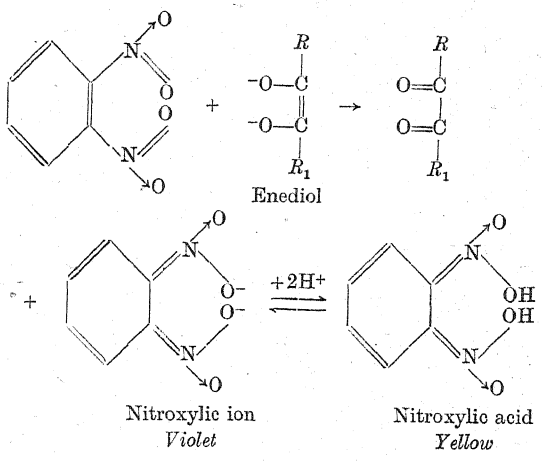
The reaction with ascorbic acid has been found to proceed on similar lines. When equimolecular proportions of ascorbic acid and *o*-dinitrobenzene interact in *N*/10 NaOH, the primary product is the violet-coloured ion of the *o*-nitroxylic acid, which on acidification is converted into the yellow form of the free acid, soluble in ether.

After recrystallization from ethanol, the acid separates out in wisps of very fine yellow needles which turn violet on exposure to NH_3. Melting-point determinations were unsatisfactory, as the acid readily dehydrates to *o*-nitro-nitrosobenzene, which is recognizable by the fact that it gives no violet colour on addition of alkalis.

The low solubility of *o*-dinitrobenzene in water (about 0·03 % at room temperature) restricts the yield of the pigment, while if the ascorbic acid be in excess at the start of the reaction the reduction proceeds beyond the nitroxylic stage.

(*d*) *Preparation of the pigment.* 100 mg. ascorbic acid (Roche or B.D.H.) were dissolved in 50 ml. Cu-free distilled water in which were suspended 100 ml. finely powdered *o*-dinitrobenzene. 1 ml. 20 % NaOH was added drop by drop, the mixture being shaken continuously. A deep violet colour appeared immediately, and became more intense as the dinitrobenzene slowly dissolved. After 12–24 hr. at room temperature, all the ascorbic acid had been oxidized, as was shown by withdrawing samples, discharging the violet colour by acetic acid, and then titrating with standard 2:6-dichlorophenolindophenol. The mixture was then filtered free from undissolved dinitrobenzene, and treated with CO_2 until the colour became orange-yellow. It was then concentrated *in vacuo*, acidity being maintained by passing in CO_2 whenever the violet colour reappeared. At the end of 2–3 days, yellow crystals of *o*-nitro-nitrosobenzene (M.P. 87–90°) began to separate out in small clusters on the surface. The yields were only about 10 % of the theoretical, the bulk of the original dinitrobenzene having undergone further reduction to a mixture of at least three different products, which separated out in a yellow crystalline crop when the solution was concentrated to a syrup.

In terms of current formulation, the primary reaction is:



(2) *Pigment formation from dehydroascorbic acid alone*

(*a*) *At* p*H 4.* 5 ml. of a dilute (0·005–0·5 %) aqueous solution of dehydroascorbic acid, adjusted to *p*H 3·5–4·5 by means of an acetate-acetic acid buffer, and gently boiled for a couple of minutes, develops a stable grass-green colour. The colour is not given by the reduced form of ascorbic acid, unless boiling is prolonged sufficiently to allow of atmospheric oxidation. Other reducing agents, such as cysteine, glutathione, the monosaccharides, uric acid and creatinine, do not give the test. Colour formation is not dependent on the presence of acetate ions, but can be observed in mixtures suitably buffered by phosphate or $(NH_4)_2SO_4$ and H_2SO_4. The green colour is stable for several hours, but may turn brown if the solution is too concentrated, the acidity insufficient, or the boiling greatly prolonged. The solution may be diluted with water for colorimetric comparison. Multivalent ions, especially Al^{+++}, Ca^{++}, Zn^{++}, and Pb^{++}, intensify the colour and change it to yellow, but accelerate the subsequent bleaching by atmospheric oxidation.

The reaction can be demonstrated by buffering 3 ml. of a 0·1 % aqueous solution of ascorbic acid by addition of about 1 g. of solid Na acetate and 1 ml. of glacial acetic acid. The mixture is then carefully oxidized by addition of 1 % iodine drop by drop, any excess of iodine being bleached by addition of a particle of thiourea or a drop of ascorbic acid solution, as it tends to destroy the pigment. After boiling the mixture for 1 min., the green colour appears and reaches a maximum within 5 min. The pigment can be extracted directly by amyl alcohol, or by ethanol or acetone after saturation of the mixture with $(NH_4)_2SO_4$. Addition of alkali converts the green into a red pigment rapidly bleached by exposure to air. Formation of the green pigment can also be observed when dehydroascorbic acid is kept in contact with concentrated HCl at room temperature, or warmed gently, but the test is best obtained by boiling at *p*H 4.

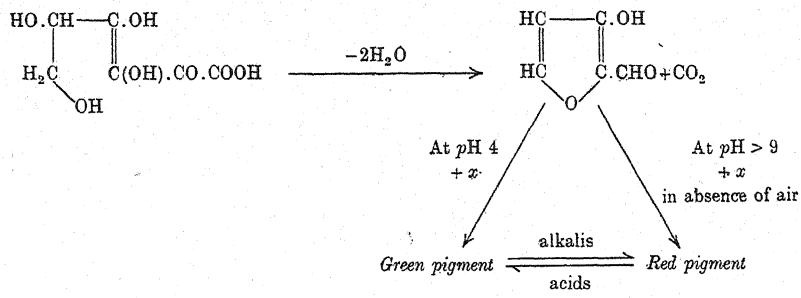
The reaction is not obviously affected by proteins, amino-acids, sugars, or the urinary solutes; and as none of the common biological compounds examined gives a similar response, the test can be used to show the presence of dehydroascorbic acid in natural sources, such as the white inner rind of citrus fruits. It can be applied to coloured solutions after acidification by acetic acid and agitation with 'norite' charcoal, which also oxidizes any ascorbic acid present. Although the intensity of the green pigment is not great, small amounts may be detected by extraction into an amyl alcohol layer.

(*b*) *At pH > 9.* When 5 ml. of a 0·1 % aqueous solution of dehydroascorbic acid, preferably in freshly boiled water, are made strongly alkaline by addition of about 1 ml. 20 % NaOH, and protected from atmospheric oxidation by a layer of toluene, a bright yellow-green colour develops within 30 min. at room temperature, and in the course of some hours slowly changes into a deep carmine red. The red pigment is rapidly bleached by exposure to air during the stage of its formation, but is stable for upwards of a week if protected from oxidation, unless the mixture be too strongly alkaline, when it changes to brown and tends to precipitate. The colour reaction is not given by reduced ascorbic acid, reducing sugars, simple ketones or other enolizable compounds. Colour formation may be hastened by gentle warming, but the pigment is then less stable. While the green precursor of the red pigment closely resembles the green pigment obtained from dehydroascorbic acid at *p*H 4, it is not identical, since it is bleached by acidification, whereas acidification of the red pigment changes it into a green indistinguishable from the *p*H 4 pigment. The red pigment in neutral solution yields a series of lakes, especially with Al and Zn, and also can be separated by adsorption on $MgCO_3$ suspensions.

(3) *Mechanism of the tests*

Pending actual isolation of the pigments, which has not yet been accomplished, the following suggestions are advanced. Since freshly prepared dehydroascorbic acid is colourless, it presumably does not contain the chromogenic 2:3-diketone system, =CH—CO—CO—*R*, but exists as the enol form, =C=C(OH)—CO—*R*. Hence, it was first assumed that the *p*H 4 green pigment was merely the true diketone form of the oxidized vitamin. This is disproved by the fact that the pigment does not yield an osazone with phenylhydrazine, and cannot be reconverted into recoverable ascorbic acid by reduction. During the process of boiling, CO_2 is evolved, which suggests that the —CO—COOH end of the chain is being decarboxylated, as would happen if a furfural derivative were being formed. That a furfural is obtained by the action of acids on dehydroascorbic acid can be demonstrated by warming some of the solution with an equal volume of concentrated HCl and a few mg. of pyrogallol, when a deep purple colour develops. Reduced ascorbic acid gives this reaction only on prolonged heating and aeration. The *p*H 4 green pigment does not give the pyrogallol reaction, nor can furfural be obtained by distillation of the mixture, which indicates that furfural formation precedes the formation of the green pigment, and enters into its construction.

Furfural can also be detected in the alkaline colour reaction by addition of acetone, which combines with it to form difurfurylacetone, stable to atmospheric oxidation and yielding a bright carmine on acidification. This can be demonstrated only in the early stages of the test.



The fact that ascorbic acid is quantitatively converted into furfural by boiling with HCl is well known [Cox, Hirst & Reynolds, 1932; Roe, 1934], while the development of a yellow colour in alkaline solutions of the vitamin has been noted [Herbert, Hirst, Percival, Reynolds & Smith, 1933], but neither the acid green pigment nor the red alkali pigment appears to have attracted attention, although the latter may be concerned in the reaction described by Kruger [1906], in which milk made strongly alkaline and kept at room temperature for several days often develops a red colour.

SUMMARY

1. A colour reaction between ascorbic acid and *o*-dinitrobenzene is described.

2. Solutions of dehydroascorbic acid spontaneously form pigments under certain conditions.

3. The specificity of these colour reactions is described, and their mechanism discussed.

We are indebted to Messrs Roche Products Ltd., for the gift of ascorbic acid, and to the Medical Research Council of Eire for the grant to one of us (E. K.) to enable this work to be done.

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Studies in Detoxication

13. THE BIOSYNTHESIS OF AMINOPHENYL- AND SULPHONAMIDOAMINOPHENYL-GLUCURONIDES IN THE RABBIT AND THEIR ACTION ON HAEMOGLOBIN *IN VITRO*

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(*Received 12 February 1943*)

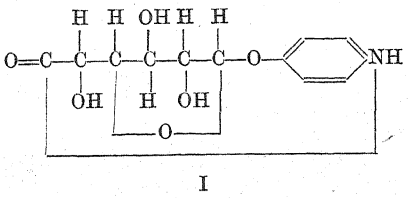
Sieberg [1914] fed 'cupferron' (ammonium salt of nitroso-β-phenylhydroxylamine) to dogs and isolated from their urine a crystalline compound, M.P. 179–180°, which he claimed to be the *lactam* form (I) of *p*-aminophenylglucuronide. He isolated the same compound from urine after the administration of β-phenylhydroxylamine. When this substance was neutralized with Na_2CO_3 and the solution kept for a day, it was able to convert a solution of haemoglobin to methaemoglobin. It should be noted that the β-glycosidic link present in this compound is stable to dilute Na_2CO_3, so that in this case formation of methaemoglobin could not be ascribed to hydrolysis of the glucuronide to *p*-aminophenol, which is a methaemoglobin-former *in vitro* and *in vivo* [Bernheim, Bernheim & Michel, 1937]. From this it would appear that the 'detoxicated' form of *p*-aminophenol could still exert a toxic effect on blood. Therefore, it was important to confirm this observation of Sieberg and to extend it to the study of the properties of the aminophenyl- and sulphonamidoaminophenylglucuronides, none of which has been prepared or described hitherto.

Aminophenylglucuronides

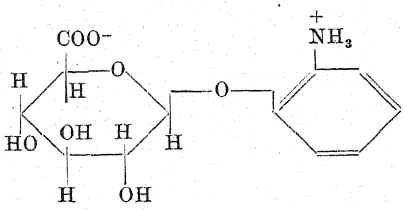
Aminophenylglucuronides are produced in the animal body in response to the administration of the aminophenols themselves, or of certain aromatic amino compounds which are oxidized or converted in some way to aminophenols. When aromatic amino compounds are oxidized *in vivo* to aminophenols, *o*- or *p*-phenols only are produced and in no case studied has *m*-aminophenol been detected.

o-Aminophenol is produced when dimethylaniline or its oxide [Horn, 1936] or acetanilide [Jaffe & Hilbert, 1888] is fed to dogs, or when salvarsan is injected into humans [Sieberg, 1916]. It is excreted in conjugation both with sulphuric and glucuronic acids, and in the rabbit some 26–29 % (dose, 0·25 g./kg.) is excreted as an ethereal sulphate [Williams, 1938]. In the present work *o*-aminophenylglucuronide has been isolated from the urine of rabbits fed with *o*-aminophenol. The yield of the crystalline glucuronide was equivalent to 25·5 % of the phenol fed and since no free aminophenol was excreted, the remainder, if excreted, must be in

other conjugated forms. *o-Aminophenyl-β-d-glucuronide* ($[\alpha]_D - 76{\cdot}1°$ in $N/10$ HCl) is a white crystalline solid which does not melt, is insoluble in cold water and ordinary organic solvents, but soluble in acid, alkali and boiling water. Its properties indicate that it is probably best formulated as an internal salt (II):



I



II

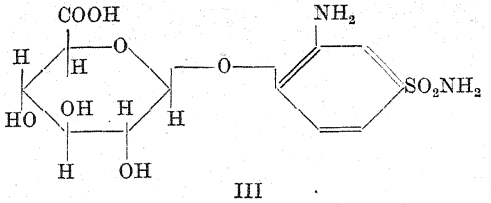
m-*Aminophenylglucuronide* ($[\alpha]_D = -94°$ in HCl) was prepared similarly. About 22 % of the *m*-aminophenol fed was isolated as the glucuronide. Williams [1938] had previously shown that 31–36 % (dose, 0·25 g./kg.) is excreted by the rabbit as an ethereal sulphate. The glucuronide is very similar in properties to the *o*-compound and is likewise probably an internal salt (cf. II).

The fate of *p*-aminophenol in the body has received more attention than that of its isomerides, because it is formed *in vivo* from aniline, acetanilide and a number of related compounds, and its formation is claimed to be responsible for the cyanosis, etc., produced by drugs containing these compounds. It has been suggested that the antipyretic action of such substances as acetanilide and *p*-ethoxy-acetanilide (phenacetin) may be due to slow formation *in vivo* of *p*-aminophenol [see Bernheim *et al.* 1937]. *p*-Aminophenol is excreted as an ethereal sulphate in the dog [Baumann & Herter, 1877–8] and in man [Mörner, 1889]. Williams [1938] showed the 19–20 % of *p*-aminophenol (dose, 0·25 g./kg.) was excreted by the rabbit as an ethereal sulphate. On feeding *p*-aminophenol to rabbits the glucuronide isolated was hydrated p-*aminophenyl-β-d-glucuronide* ($C_{12}H_{15}NO_7$, H_2O) and not Sieberg's compound (I). Like Sieberg's compound, however, *p*-aminophenylglucuronide neutralized with Na_2CO_3 converts haemoglobin to methaemoglobin *in vitro*.

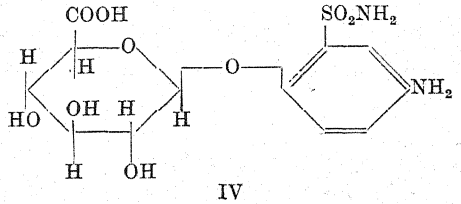
p-Aminophenylglucuronide was similar in many properties to its *o*- and *m*-isomers, but differed in that it showed a definite melting-point and contained water of crystallization. The amount of the glucuronide isolated was about 8 % of the phenol fed, and unlike the *o*- and *m*-compounds, free *p*-aminophenol was present in the urine.

Sulphonamidoaminophenylglucuronides

It was suggested [Thorpe, Williams & Shelswell, 1941] that 3-hydroxy-4-aminobenzenesulphonamide might be an oxidation product of sulphanilamide *in vivo*. Shelswell & Williams [1940] showed that sulphanilamide increased the ethereal sulphate output of rabbits, thus indicating the probable formation of a phenol from sulphanilamide in the animal [cf. also Sammons, Shelswell & Williams, 1941; Williams, 1941]. Such an aminophenolsulphonamide could be excreted as a conjugated glucuronide (cf. sulphapyridine which increases the glucuronic acid output in rats [Scudi & Robinson, 1941]). Aminophenolsulphonamides were therefore fed and an attempt was made to isolate the resulting sulphonamidoaminophenylglucuronides. 4-Hydroxy-3-amino-, 6-hydroxy-3-amino- [Williams, 1942] and 3-hydroxy-4-aminobenzenesulphonamide [Thorpe & Williams, 1941] were fed and crystalline glucuronides were isolated in the first two cases, but in the third, although a glucuronide was formed it was not obtained crystalline but as an amorphous Ba salt which has not yet been purified. The crystalline compounds were 4-*sulphonamido*-2-*aminophenylglucuronide* (III), M.P. 150° and $[\alpha]_D - 67{\cdot}7°$, and 2-*sulphonamido*-4-*aminophenylglucuronide* (IV) which does not melt and shows $[\alpha]_D - 76{\cdot}9°$. Their properties were very similar to those of the aminophenylglucuronides and when neutralized with Na_2CO_3 they converted haemoglobin to methaemoglobin *in vitro*:



III



IV

The action of the above glucuronides on haemoglobin in vitro

It was found that the *o*- and *p*-aminophenylglucuronides when neutralized with Na_2CO_3 converted

a solution of oxyhaemoglobin* to methaemoglobin, the change being observed spectroscopically. The tests were carried out for a period up to 6 hr., but as a rule the methaemoglobin-formers produced their effect in 2–3 hr. No change in the spectrum of the control solution occurred in this time. Fairley [1940] found that a concentrated solution of human haemoglobin incubated at 37° began to form methaemoglobin spontaneously within 20 hr., but the present experiments were not long enough for this. The results of the present experiments are shown in Tables 1 and 2.

The *o*-compound appeared to be several times more effective than the *para*-. A slight formation of methaemoglobin was also observed in the case of the *m*-compound, although *m*-aminophenol itself was quite ineffective in this respect. The neutralized solutions of the glucuronides were quite stable since no free aminophenols were obtained from them on continuous extraction with ether, although if present they would readily have been extracted. If red corpuscles contained β-glucuronidase, hydrolysis of these glucuronides might have occurred. Whilst extracts of ox and dog kidney, liver and spleen hydrolysed conjugated glucuronides, the latter were unaffected when incubated with ox or dog blood [Sera, 1915]. Oshima [1934] and Fishman [1940] make no reference to the occurrence of β-glucuronidase in blood. It is unlikely, therefore, that the aminophenylglucuronides would undergo spontaneous or enzymic hydrolysis under the conditions of the present experiments. It is, however, significant that the most effective methaemoglobin-formers are the *o*- and *p*-compounds, i.e. derivatives of *o*- and *p*-aminophenols which themselves can form iminoquinones. The formation of iminoquinones does not appear possible with the aminophenylglucuronides.

The sulphonamidoaminophenylglucuronides (III and IV) were also methaemoglobin-formers, as were also the corresponding aminophenolsulphonamides and particularly the aminophenolsulphonic acids. The *o*-, *m*- and *p*-aminobenzenesulphonamides were ineffective *in vitro*, although it is well known that sulphanilamide forms methaemoglobin *in vivo*, probably due to its conversion to an aminophenolsulphonamide.

The present findings, therefore, confirm and extend Sieberg's original observation. *o*- and *p*-aminophenylglucuronides and the sulphonamido-*o*- and -*p*-aminophenylglucuronides convert haemoglobin to methaemoglobin *in vitro*. Discussion of the significance of these results, and their translation to *in vivo* conditions, however, must await further investigation.

* Lysed human red cells or solutions of Hb washed free from plasma were used in these experiments in order to avoid a possible complication due to methaemalbumin (pseudo-methaemoglobin) formation which may occur when whole human blood is used [see Fairley, 1938; 1940; 1941].

EXPERIMENTAL

Isolation of the aminophenylglucuronides

o-*Aminophenylglucuronide.* Six rabbits (2·5 kg.) were each given 1 g. of *o*-aminophenol with water by stomach tube. In about 1 hr. the excreted urine showed the presence of conjugated aminophenol and increased amounts of glucuronic acid. In 3·5 hr. 300 ml. of urine collected which gave an intense naphthoresorcinol reaction, a red colour with nitrous acid and dimethyl-α-naphthylamine (diazo reaction), but did not reduce ammoniacal $AgNO_3$. The urine was acidified with a few drops of glacial acetic acid and treated with saturated normal lead acetate until no further precipitate was formed. The filtrate was neutralized with NH_3 and treated with excess saturated basic lead acetate solution. The precipitate was centrifuged, washed twice on the centrifuge with water, and the lead salt suspended in water and decomposed with H_2S. The filtrate from the PbS was concentrated *in vacuo* at 45–50°, when the o-*aminophenylglucuronide* began to separate as a white crystalline solid, and finally taken to dryness. The white residue was shaken with ethanol and the whole filtered. The glucuronide was then washed with ethanol and ether (yield 3·3 g.). A second portion of urine (210 ml.), collected during the 2 hr. following the first, yielded 0·7 g. of the glucuronide. The total yield for 5 hr. was 4 g. or 25·5% of the aminophenol fed. Later specimens of urine contained very little of the glucuronide.

The glucuronide was recrystallized from a large volume of hot water and formed cigar-shaped platelets which blackened without melting on heating to 300°. It was insoluble in cold water, ethanol, ether and most organic solvents, sparingly soluble in hot water, but soluble in acid and alkali. It showed $[\alpha]_D - 76{\cdot}1°$ ($c = 1$ in $N/10$ HCl). Found: C, 50·7; H, 5·35; N, 4·9%. $C_{12}H_{15}O_7N$ requires C, 50·5; H, 5·3; N, 4·9%.

m-*Aminophenylglucuronide.* Six rabbits were each given 1 g. of *m*-aminophenol with water by stomach tube. The urine was worked up in two portions as described above for the *ortho* compound. The first portion (375 ml.), collected during 3 hr., yielded 2·4 g. of m-*aminophenylglucuronide*; the second, collected during the next 4 hr., gave 1·03 g.: total yield 3·45 g. or 21·9% of the aminophenol fed. The urine gave a bluish red diazo reaction, an intense naphthoresorcinol reaction and was non-reducing.

The glucuronide recrystallized from hot water as leaflets, which blackened without melting on heating to 300°. Its solubility was similar to that of the *ortho* compound and it showed $[\alpha]_D^{14°} - 94°$ ($c = 0{\cdot}95$ in $N/12$ HCl). Found: C, 50·1; H, 5·3; N, 4·9%. $C_{12}H_{15}O_7N$ requires C, 50·5; H, 5·3; N, 4·9%.

p-*Aminophenylglucuronide.* *p*-Aminophenol (1 g.) was fed by stomach tube with water to each of six rabbits. The urine was collected in two portions as before. The first (340 ml.), collected after 3½ hr., was dark but clear. It gave a purple diazo reaction and positive indophenol and naphthoresorcinol reactions. It reduced ammoniacal $AgNO_3$ in the cold, and an ether extract gave the indophenol reaction, indicating the presence of free *p*-aminophenol in the urine (*p*-aminophenylglucuronide is insoluble in ether).

The urine was worked up as in the other two cases and the resulting p-*aminophenylglucuronide* (1·4 g.) was recrystallized from hot water, from which it formed needles, M.P. 213° (decomp.). The second batch of urine was collected up to 24 hr. after feeding the phenol and although some glucuronide was present, it could not be obtained crystalline. The total crystalline material isolated was 1·4 g. or 8·4% of the aminophenol fed. It was insoluble in cold water, ethanol or ether and the analytical figures showed it to contain 1 mol. of water of crystallization, which was not lost at 104° after several hours. It showed $[\alpha]_D^{22°} - 82·7°$ ($c=1$ in $N/10$ HCl). Found: C, 47·6; H, 5·55; N, 4·45%. $C_{12}H_{15}O_7N$, H_2O requires C, 47·5; H, 5·6; N, 4·6%.

Isolation of sulphonamidoaminophenylglucuronides

4-Sulphonamido-2-aminophenylglucuronide. Rabbits were given by stomach tube with water, 0·4–0·6 g./kg. of *o*-aminophenol-*p*-sulphonamide. The urine passed within the first hour gave a red colour in the diazo reaction and a positive naphthoresorcinol reaction, thus indicating excretion of the conjugated glucuronide of the phenol. The urine was collected for 24 hr. In one experiment, for example, a total of 4·4 g. of the sulphonamide was fed and 670 ml. urine were collected in 24 hr. The basic lead precipitate was prepared as usual, the lead removed by H_2S, and the filtrate evaporated to dryness *in vacuo*. The residual syrup taken up in ethanol and again concentrated at 45° crystallized only after the addition of crystals prepared from a small portion dissolved in a little ethanol to which an excess of light petroleum had been added. The yield of glucuronide was 0·42 g. from 4·4 g. of the sulphonamide fed (other preparations yielded 0·2 and 0·21 g. from 3 and 4·2 g. of the sulphonamide, respectively).

4-Sulphonamido-2-aminophenylglucuronide formed small rectangular notched plates from hot water, M.P. 150° (decomp. to an orange froth). It was almost insoluble in cold water, insoluble in ethanol and ether, but soluble in hot water, acid and alkali. It showed $[\alpha]_D^{20°} - 67·7°$ ($c=2$ in water containing 1 equiv. Na_2CO_3, i.e. enough alkali to form a monosodium salt). Found: C, 35·7; H, 5·1; N, 6·6; S, 7·4%. $C_{12}H_{16}O_9N_2S$, $2\frac{1}{2}H_2O$ requires C, 35·2; H, 5·1; N, 6·8; S, 7·8%.

2-Sulphonamido-4-aminophenylglucuronide. Three rabbits* were each given 1·3 g. (0·75 g./kg.) of *p*-aminophenol-*o*-sulphonamide [Williams, 1942]. The urine (590 ml.), which was blackish in colour, was collected for 24 hr. and worked up as usual. The non-crystalline concentrate from the basic lead precipitate was dissolved in absolute ethanol, charcoaled, filtered and then concentrated *in vacuo* to a syrup. The solution in aqueous ethanol would not crystallize without the addition of a trace of crystalline 4-sulphonamido-2-aminophenylglucuronide, and 0·62 g. of crystalline 2-*sulphonamido*-4-*aminophenylglucuronide* was thus obtained. It formed small thick rectangular plates (not notched like its isomeride) from hot water. It did not melt up to 300°, but turned red at 140° and black at 190°. It was almost insoluble in cold water and insoluble in ethanol and ether. It showed $[\alpha]_D^{21°} - 76·9°$ ($c=1$ in water as monosodium salt). Found: C, 35·4; H, 5·1; N, 6·35; S, 7·65%. $C_{12}H_{16}O_9N_2S$, $2\frac{1}{2}H_2O$ requires C, 35·2; H, 5·1; N, 6·8; S, 7·8%.

The action of o-, m- *and* p-*aminophenylglucuronides on haemoglobin solutions*

Human red cells from the blood bank were used. The blood had been treated with Na citrate and allowed to stand for 7 days at a low temperature. Then the plasma had been poured off and the residual red cells were used in these tests. In the first case, 10 ml. of the red cells as obtained from the blood bank were diluted to 200 ml. with water. In the second case, the red cells were washed three times in the centrifuge with 0·9% NaCl, haemolysed by shaking with asbestos and filtered through a thick layer of cotton-wool. 5 ml. of the filtrate were diluted to 200 ml. The results of the experiments were essentially the same for both solutions of haemoglobin. To each of a series of small test tubes containing 3 ml. of the haemoglobin solution was added the required amount of an aqueous solution

Table 1. *Action of* o-, m- *and* p-*aminophenylglucuronides on haemoglobin*

Compound	mg. compound ml. Hb solution	Time (hr.) 0	0·75	2	3	5–6
Control	0	–	–	–	–	–
o-	3	–	–	+	+ +	.
m-		–	–	–	–	–
p-		–	–	–	–	+
o-	6	–	+	+ +	+ + +	.
m-		–	–	–	–	–
p-		–	.	+	+	+
o-	9	–	+	+ + +	+ + + +	.
m-		–	–	–	–	+
p-		–	.	+	.	+ +
o-	12	–	+ +	+ + + +	+ + + +	.
m-		–	–	–	–	+
p-		–	.	+ +	.	+ + +

– Oxyhaemoglobin spectrum unchanged.
\+ Faint methaemoglobin line in red.
\+ + Definite methaemoglobin line in red.
\+ + + Strong methaemoglobin line in red.
\+ + + + Stronger methaemoglobin line in red.
Blank No observation at that time.

* Two of these rabbits died 2 days later and the third, being obviously ill, was killed. In all these animals the liver was found to be pulpy and friable. One day after administration of the sulphonamide all three animals salivated profusely.

of the Na salt of the aminophenylglucuronide and water to make up to 5 ml. The tests were set up in duplicate and incubated, with the appropriate controls, at 37°. The tubes were periodically examined with a spectroscope for methaemoglobin. Initially all solutions gave a well-marked oxyhaemoglobin absorption spectrum. The results are given in Table 1.

The aqueous solutions of the Na salts of all three aminophenylglucuronides were subjected to continuous extraction with ether for several hours, but in no case could free aminophenols be detected in the extract. Added free aminophenols could easily be extracted under these conditions.

2·6 mg./ml.). To each tube were then added 4 ml. haemoglobin solution. Hence the compounds used were present in amounts equivalent to 1 mg. aminophenol/ml. The tubes were incubated and the spectra observed periodically as before.

Table 2. *On methaemoglobin formation* in vitro

Compound	Mg. compound 5 ml. sol.	Time (hr.) 1	1·5	2	3	5·5
Control (water)	0	−	−	−	−	−
Control (Na_2CO_3 solution)	0	−	−	−	−	−
Glucurone	8	−	−	−	−	−
Sulphanilamide	8	−	−	−	−	−
Metanilamide	8	−	−	−	−	−
Orthanilamide	8	−	−	−	−	−
Acetylsulphanilamide	10	−	−	−	−	−
l-Menthylglucuronide	17	−	−	−	−	+
Euxanthic acid	18	−	−	+	+	+
o-Aminophenylglucuronide	14	−	+ +	+ + +	+ + + +	+ + + +
m-Aminophenylglucuronide	14	−	−	+	+	+
p-Aminophenylglucuronide	15	−	+	+	+ +	+ + +
4-Hydroxy-3-aminobenzenesulphonamide	9	−	−	+	+	+ +
3-Hydroxy-4-aminobenzenesulphonamide	9	−	−	+	+	+
3-Hydroxy-4-aminobenzenesulphonic acid	9	+ +	+ +	+ + + +	+ + + +	+ + + +
6-Hydroxy-3-aminobenzenesulphonamide	9	−	+	+	Solution discoloured	
6-Hydroxy-3-aminobenzenesulphonic acid	9	+ +	+ +	+ + + +	+ + + +	+ + + +
4-Sulphonamido-2-aminophenylglucuronide	17	−	+	+	+	+
2-Sulphonamido-4-aminophenylglucuronide	17	−	+	+	+	+ +
o- and *p*-Aminophenols	5	Solutions discoloured				
m-Aminophenol	5	−	−	−	−	−

\- Oxyhaemoglobin spectrum unchanged.
\+ Methaemoglobin spectrum just visible.
\+ + Methaemoglobin spectrum definite.
\+ + + Methaemoglobin spectrum strong.
\+ + + + Methaemoglobin spectrum very strong.

The sulphonamidoaminophenylglucuronides and some other compounds were also tested (Table 2). Each compound, in amount equimolar to that of aminophenol (5 mg.), was weighed into a small test tube, each tube being in duplicate. The compound was dissolved with slight warming, if necessary, in 1 ml. Na_2CO_3 containing 1 equiv. (i.e.

SUMMARY

1. The preparation is described of *o*-, *m*- and *p*-aminophenylglucuronide and 2-sulphonamido-4-amino- and 4-sulphonamido-2-aminophenylglucuronides by feeding the corresponding aminophenols to rabbits.

2. It has been shown that the *o*- and *p*-aminophenylglucuronides are methaemoglobin-formers *in vitro*. The sulphonamidoaminophenylglucuronides also caused methaemoglobin-formation, but less powerfully than the aminophenylglucuronides.

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The Effect of Citrate on the Rotation of the Molybdate Complexes of Malate, Citramalate and Isocitrate

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Extracts of liver and muscle convert citric acid into laevo-rotatory isocitric acid [Martius, 1938]. As the rotation of isocitric acid, like that of other α-hydroxy acids, is much increased by $(NH_4)_2MoO_4$*, the polarimeter can be used to measure low concentrations of isocitric acid in tissue extracts [Martius, 1938; Jacobsohn, 1940].

On applying this method to plant extracts we met with an unexpected complication. Aqueous extracts of rhubarb petioles showed a dextrorotation with molybdate, owing to the presence of *l*(−)malic acid. When the extracts were mixed with citrate the dextrorotation increased very considerably, e.g. from +1·75° to +3·25°. Investigation of this effect showed that the increase was not caused by an enzymic process; it proved to be due to an effect of citrate on the dextrorotation of the *l*(−)malate-molybdate complex. Under the test conditions $[\alpha]_D^{20°}$ was +716° for *l*(−)malic acid in the presence of $(NH_4)_2MoO_4$ and +1340° for *l*(−)malic acid in the presence of $(NH_4)_2MoO_4$ and citrate. Citrate was found also to change the rotation of the molybdate complexes of two substituted malic acids, viz. citramalic and isocitric acids.

The effect of citrate on the rotation of the malate-molybdate complex has already been observed by Auerbach & Krüger [1923] who realized that citrate interferes with the polarimetric determination of malic acid. Obviously citrate also interferes with the Martius polarimetric isocitric acid estimation. Isocitric acid is frequently accompanied in biological material by citric acid and many of the data calculated from polarimetric readings by previous authors therefore require revision.

EXPERIMENTAL

Materials. Samples of *l*(−)malic acid obtained from Messrs Schering-Kahlbaum and from Dr W. Fraenkel, Factory M3, Treforest Estate, near Cardiff, were used, both giving identical results. Na-*l*(−)α-hydroxyglutarate was prepared according to Fischer & Moreschi [1912], citramalic acid according to Michael & Tissot [1892]; it was resolved with brucine according to Marckwald & Axelrod [1899]; the sample of *l*(+)lactic acid used was obtained from Messrs Schuchardt. Pure optically active isocitric acid was not available; a solution of the active acid was prepared by bacterial resolution of Na isocitrate synthesized according to Fittig & Miller [1889] and Wislicenus & Nassauer [1895].

Calculations. The specific rotation was calculated according to the formula $[\alpha]_D = 100/l \times c$. The values of *c* refer to the concentrations of the free organic acid (g. %), and not to the concentration of the molybdate complexes.

* This formula is used, for brevity's sake, to denote commercial ammonium molybdate.

RESULTS

Effect of citrate on the rotation of extracts of rhubarb petioles

Petioles of forced rhubarb were minced and then ground to a pulp in a mechanical mortar; half volume of H_2O was added during the grinding. Ten ml. pulp, 5 ml. *M* Na_3-citrate and 1 ml. 3 *M* acetate buffer of *p*H 4·0 were mixed and allowed to stand for 19 hr. at 18°, octanol serving as an antiseptic. Then 1·6 ml. glacial acetic acid and 14·4 ml. freshly prepared 29 % $(NH_4)_2MoO_4$ were added, the insoluble material filtered off and the filtrate cleared with a small quantity of charcoal.

A control tube contained water instead of the citrate solution. The polarimeter readings (2 dm. tube, Na-lamp) were: +3·25°; control, +1·75° (18°).

The results were independent of the period of incubation but approximately proportional to the quantity of pulp used. The effect of citrate could not therefore be attributed to the action of an enzyme.

Effect of citrate on the rotation of the molybdate complex of malic acid and other hydroxy acids

The rotation of *l*(−)malic acid (in the presence of $(NH_4)_2MoO_4$) is raised by citrate (Table 1) in the same proportion and in the same direction as is the rotation of the rhubarb extract, the increase being 85·5 % in the case of rhubarb extract and 87 % in the case of malic acid. The effect of citrate on the rotation of rhubarb extract can thus be explained as an effect on the rotation of malate (which is known to occur in rhubarb). The rotation of the molybdate complexes of lactic, tartaric and α-hydroxyglutaric acid are also changed by citrate but these effects are small as compared with the effect of the rotation of malic acid.

Citramalic acid-(methyl malic acid) behaves similarly to malic acid. The rotations of the molybdate complexes of malic and citramalic acids are almost identical, both in the absence and in the presence of citrate (Table 1).

Table 1. *Rotatory power of hydroxy acids in the presence of $(NH_4)_2MoO_4$ and other substances*

(2·5 ml. hydroxy acid solution; 2·5 ml. solution of the 'second substance'; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 18°; 1 dm. tube.)

Hydroxy acid (final conc.)	'Second substance' (final conc.)	α_D (°)	$[\alpha]_D$ (°)
l(−)Malic acid (0·0187 *M* = 0·25%)	—	+1·79	+ 716
	Na_3-citrate (0·25 *M*)	+3·35	+1340
l(+)Lactic acid (0·25 *M*)	—	−1·31	− 58
	Na_3-citrate (0·25 *M*)	−1·28	− 56
l(+)Tartaric acid (0·0334 *M*)	—	+3·30	+ 660
	Na_3-citrate (0·25 *M*)	+3·48	+ 696
l(−)α-Hydroxyglutaric acid (0·026 *M*)	—	−0·39	− 101
	Na_3-citrate (0·25 *M*)	−0·30	− 78
l(−)Citramalic acid (0·01 *M*)	—	−1·11	− 748
	Na_3-citrate (0·25 *M*)	−1·98	−1335
l(−)Malic acid (0·0187 *M*)	Na_2-racemate (0·25 *M*)	+1·21	+ 484
	dl-Na-lactate (0·25 *M*)	+1·31	+ 524
	dl-Na-β-hydroxybutyrate (0·25 *M*)	+1·73	+ 692
	Glycerol (0·6 *M*)	+1·44	+ 576
	Na_3-*trans*-aconitate (0·25 *M*)	+1·79	+ 716
	dl-Na_2-citramalate (0·25 *M*)	+1·84	+ 736

Effects of optically inactive hydroxy compounds other than citrate. Racemic acid, *dl*-lactic acid, *dl*-β-hydroxybutyric acid or glycerol all depress the rotation of the malate-molybdate complex, whilst *trans*-aconitic acid and *dl*-citramalic acid have no definite effect (Table 1).

Effect of citrate concentration. A concentration of citrate equivalent to that of malate already increases the rotation of malic acid considerably, but the maximum effect requires, under the test conditions, a citrate concentration of about 0·1 molar, i.e. about 5 times the concentration of malate. Very high concentrations of citrate (1 *M*) abolish the dextrorotation of the malate-molybdate complex, presumably by competitive reaction with the available molybdate (Table 2).

Table 2. *Effect of citrate concentration on the rotation of the malate-molybdate complex*

(2·5 ml. 1% *l*(−)malic acid solution—final malic acid conc.: 0·0187 *M*; 2·5 ml. Na_3-citrate solution diluted to different conc.; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 15°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)	$[\alpha]_D$ (°)
1·0	−0·08	—
0·5	+0·81	+ 324
0·25	+3·37	+1340
0·125	+3·36	+1335
0·0625	+3·16	+1260
0·03125	+2·82	+1130
0·01563	+2·48	+ 990
None	+1·79	+ 716

Effect of molybdate concentration. The rotation of the malate-molybdate complex does not significantly change when the concentration of $(NH_4)_2MoO_4$ varies between 3 and 13·5%. With citrate there is a diminished rotation when the molybdate concentration falls below 9%. Below this concentration the available molybdate is probably insufficient to 'saturate' both citrate and malate (Table 3).

Table 3. *Effect of $(NH_4)_2MoO_4$ concentration on the rotation of* l(−)*malic acid in the presence of citrate*

(2·5 ml. 1% *l*(−)malic acid; 0·5 ml. glacial acetic acid; 2·5 ml. 1 *M* Na_3-citrate or water; 4·5 ml. $(NH_4)_2MoO_4$ solution of varying strength; 15°; 1 dm. tube.)

$(NH_4)_2MoO_4$ (final conc.) (%)	α_D without citrate (°)	α_D with citrate (°)
13·5	+1·82	+3·50
9·0	+1·85	+3·37
6·0	+1·87	+0·96
3·0	+1·85	−0·08

Effect of the malic acid concentration. The specific rotation of the malate-molybdate-citrate complex is virtually constant when the malic acid concentration rises from 0·015 to 0·25% (Table 4).

Table 4. *Effect of malic acid concentration on the rotation of the malate-molybdate-citrate complex*

(2·5 ml. malic acid solution; 2·5 ml. Na_3-citrate 1 *M*; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 17°; 1 dm. tube.)

Malic acid (final conc.) (%)	α_D (°)	$[\alpha]_D$ (°)
0·25	+3·34	+1335
0·125	+1·76	+1407
0·0625	+0·90	+1440
0·03125	+0·45	+1440
0·01563	+0·23	+1470

Effect of temperature on the rotation of the malate-molybdate-citrate complex. At 6·5°, 17·5° and 37·5° we find for α_D +3·44°, +3·34° and +3·03° (under the conditions stated in Table 4).

Effect of citrate on the rotation of the citramalate-molybdate complex. The effect of citrate on the rotation of the citramalate-molybdate complex is similar in magnitude to the effect on the malate complex (Table 5).

Table 5. *Effect of citrate on the rotation of the citramalate-molybdate complex*

(3 ml. 0·445% (−)citramalic acid solution—final citramalic acid conc. 0·01 *M*; 2 ml. Na_3-citrate solutions of varying conc.; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 22°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)	$[\alpha]_D$ (°)
0·2	−2·04	−1380
0·1	−2·00	−1350
0·05	−1·90	−1280
0·025	−1·73	−1170
0·0125	−1·55	−1040
0·00625	−1·41	− 452
0·00313	−1·27	− 856
None	−1·11	− 748

Experiments with isocitric acid

Since pure optically active isocitric acid was not available a solution of the active acid was prepared by bacterial resolution of synthetic Na_3-isocitrate. *Pseudomonas pyocyanea, Aerobacter aerogenes* or *Bact. prodigiosum* all resolve isocitrate [Martius, 1938]. The culture medium contained 1 g. KH_2PO_4, 1 g. Na_2HPO_4, 5 g. NH_4Cl, 0·2 g. $MgSO_4$, $7H_2O$, 0·05 g. $CaCl_2$ and 9·6 g. isocitric acid (as Na salt) per litre. 100 ml. medium were sterilized in Roux culture flasks of 1 l. capacity and inoculated with one of the above organisms. The flasks were laid out flat in order to facilitate aeration. When a heavy inoculum was used the maximum rotation was reached after 3 or 4 days' incubation at 37°, α_D of the medium being +1·69° (2 dm. tube; 5 ml. medium diluted with 0·5 ml. glacial acetic acid and 4·5 ml. 29% molybdate, filtered).

Like muscle tissue [Martius, 1938], the bacteria tested metabolize 50% of synthetic isocitrate. This is shown by the observation that a washed suspension of *Aerobacter aerogenes* (*p*H 6·8; 40°) absorbed 438 μl. extra O_2 on addition of 0·1 ml. 0·05 *M* citrate, and 226 μl. on addition of the same amount of synthetic isocitrate (calc. for complete oxidation: 504 μl.). The fact that less than the calculated amount of O_2 was absorbed was presumably due to the synthesis of cell material from the substrate [cf. Clifton, 1937].

For the following experiments 300 ml. medium were incubated for 3 days with *Aerobacter aerogenes*; the culture was then concentrated on the steam-bath to about 50 ml., and filtered after addition of charcoal. The effect of citrate on the rotation of the isocitrate solution in the presence of molybdate is shown in Tables 6 and 7. The increase caused by 0·1 *M* citrate varies between 40% and 120%.

Table 6. *Rotation of isocitrate solutions of varying concentrations in presence of citrate and molybdate*

(10 ml. isocitrate solution; 1·0 ml. glacial acetic acid; 9 ml. 29% $(NH_4)_2MoO_4$; 2 dm. tube; for the measurements recorded in the last two columns the isocitrate stock solution was diluted with Na_3-citrate solutions; the citrate concentration stated refers to the final concentration.)

Relative conc. of isocitrate (Data are no. of times stock solution was diluted)	α_D (°)		
	No citrate added	0·1 *M* citrate	0·2 *M* citrate
Undiluted	+9·64	—	—
2	+5·62	+7·86	+6·44
4	+2·72	+4·52	+3·96
8	+1·23	+2·47	+2·12
16	+0·58	+1·26	+1·12
32	+0·30	+0·66	—

Table 7. *Effect of citrate concentration on the rotation of the isocitrate-molybdate complex*

(Stock solution of isocitrate diluted with 3 vol. H_2O; 4 ml. diluted stock solution; 1 ml. citrate; 0·5 ml. glacial acetic acid; 4·5 ml. 29% molybdate; 20°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)
0·1	+1·89
0·05	+1·77
0·025	+1·58
0·0125	+1·41
0·00625	+1·27
0·003125	+1·11
None	+1·04

Equilibrium between citric, isocitric and cis-aconitic acids

Martius [1938] calculated from polarimetric readings that the equilibrium mixture of the three tricarboxylic acids in the presence of aconitase contains 9·4% isocitric acid. The calculation was based on the assumption that the specific rotation of isocitric acid in the presence of molybdate is −428°. Since this value applies to pure isocitric acid solutions it is no doubt too low for the conditions of Martius's experiments, where about 0·09 *M* citrate was present.

The data presented in Tables 6 and 7 permit an approximate estimate of the specific rotation of isocitric acid in the presence of citrate. Under the conditions of Martius's measurements the increase brought about by citrate is about 82%; this would bring $[\alpha]_D$ to −780°. Using this figure for the recalculation of Martius's data we obtain a value of 5·15% isocitric acid in the equilibrium mixture.

A number of further measurements of the final rotation of solutions in which the enzymic equilibrium between the three tricarboxylic acids was established, are shown in Table 8. The solutions

contained 0·2 *M* Na citrate, varying quantities of buffer, minced tissue and a drop of octanol. The mixture was incubated at 38°. For the determination of the rotation 10 ml. were acidified with 1 ml. glacial acetic acid, ice-cooled, and mixed with 9 ml. 29 % $(NH_4)_2MoO_4$. The solution was filtered after adding a small quantity of charcoal, and rapidly examined in 2 dm. tubes. Ice-cooling was necessary in order to prevent the development of the blue colour of the reduced phosphomolybdate complex. Under the conditions of the measurements the concentration of citrate was about 0·09 *M*.

Table 8. *Equilibrium at 38° between citrate, isocitrate and* cis-*aconitate in the presence of aconitase*

(Unless otherwise stated the tissue concentration in the enzyme preparation was 3·0 g. of fresh tissue in 50 ml.; the buffer consisted of phosphate except in the one instance indicated, in which $NaHCO_3/CO_2$ was used.)

Buffer: Final conc. *M*	Buffer: *p*H	Period of incubation hr.	$\alpha_D^{18°}$ (° for 2 dm.): Without citrate	With citrate	Difference	*cis*-Aconitic acid %*
		(*a*) Enzyme from guinea-pig liver				
0·030	7·4	18†	+0·06	−1·74	−1·80	4·08
0·025	6·8	17	+0·13	−1·70	−1·83	3·52
0·025	7·1	17	+0·12	−1·68	−1·80	—
0·025	6·8	17	+0·11	−1·79	−1·90	4·19
0·025	6·8	17	+0·12	−1·72	−1·84	5·04
0·050	6·8	17	+0·02	−1·73	−1·75	4·26
0·060‡	6·4‡	24	−0·14	−1·81	−1·67	—
0·025	6·8	17	+0·13	−1·77	−1·90	4·73
0·025	6·8	17	+0·16	−1·72	−1·88	4·22
		(*b*) Enzyme from guinea-pig muscle				
0·010	6·8	41	−0·06	−1·82	−1·76	4·33
		(*c*) Enzyme from pigeon breast muscle				
0·025	6·8	22	−0·01	−1·77	−1·76	4·22

* The figures in this column are

$$\frac{\text{molecules of } cis\text{-aconitic acid found}}{\text{molecules of citric acid added}} \times 100.$$

† Tissue conc. = 1·5 g./50 ml.
‡ Buffer = $NaHCO_3/CO_2$'

The rotations observed were a little higher than those reported by Martius [1938] and by Jacobsohn, Soares & Tapadinhas [1940]. The average of six measurements of α_D under the same conditions (*p*H 6·8; 0·025 *M* phosphate) is −1·85°. The deviations from this mean are considerably greater than the experimental error (which is ±0·04°). They may be connected with the rotation of the control. As the crude tissue extracts used in our experiments contained cathepsin and amylase, optically active substances may have been formed during the incubation from protein or carbohydrates. It is possible that the high concentration of citrate affects these enzymes and this would invalidate the control experiments. More accurate estimations of the rotation caused by isocitric acid would require the use of a purified aconitase free from other enzymes and their substrates.

On the basis of the assumption that $[\alpha]_D$ for the experimental conditions is −780°, the rotation of −1·85° indicates that 6·2 % of the citric acid was converted into isocitric acid. The average proportion of aconitic acid (determined according to Johnson [1939]) was 4·3 %. This leaves 89·5 % for citric acid. Determinations of citric acid according to Pucher, Sherman & Vickery [1936] and Kometiani [1931] agreed with this calculation within the limits of the error of the methods, which proved to be about ±10 %.

The values for *cis*-aconitic acid are in accordance with those previously reported from this laboratory by Johnson [1939]. Johnson calculated the isocitric acid concentration of the equilibrium mixture by deducting the sum of the aconitic and citric acid recovered from the amount of citric acid added. In the light of more recent experience with the citric acid determination according to Pucher *et al.* [1936] [see also Dickens, 1941], it is probable that Johnson's recovery of citrate was somewhat incomplete. We find that the yield is frequently low when permanganate is added rapidly to the citrate solution and we therefore now add the reagent gradually, as recommended by Reichard [1934]. This precaution was not observed in Johnson's experiments; his citrate values therefore were probably too low, and the calculated isocitrate values too high.

Change of *p*H from 6·8 to 7·4, or the substitution of phosphate buffer by bicarbonate buffer, does not appreciably affect the equilibrium (Table 8). Confirming Jacobsohn's [1940] observation we find that addition of $MgCl_2$ (0·12 *M*) reduces the concentration of isocitric acid in the equilibrium mixture from 6·2 to 2·6 % (assuming $[\alpha]_D$ to be −780°). Under the same conditions (*p*H 6·8; 0·025 *M* phosphate buffer) the concentration of *cis*-aconitic acid fell from 4·2 to 1·6 %. $MgCl_2$ thus shifts the equilibrium in favour of citrate, presumably because of the formation of a Mg-citrate complex [see Hastings, McLean, Eichelberger, Hall & da Costa, 1934; Nordbö, 1938] which results in a lowering of the concentration of 'free' citrate in the system.

Polarimetric determination of malic and isocitric acids

To overcome the difficulty arising from the interference by citrate with the rotation of the molybdate complexes, we add citrate to the solution in quantities sufficient to bring the citrate concentration to about 0·1 *M*; 4 ml. of the solution to be examined (which should be neutral) are mixed with 1 ml. *M* Na_3-citrate, 0·5 ml. acetic acid and 4·5 ml. 29 % $(NH_4)_2MoO_4$. The data recorded in Tables 4 and 6 show that the rotations of malic and isocitric acids under these conditions are approximately proportional to the hydroxy acid concentration. This procedure has the further advantage of increasing the sensitivity of the polarimetric method.

SUMMARY

1. Citrate increases the rotation of the molybdate complexes of malic acid (as already observed by Auerbach & Krüger [1923]), of citramalic acid and of isocitric acid. The increase can amount to more than 100%. The magnitude of the effect under varying conditions is investigated.

2. The effect of citrate must be taken into account when the above acids are determined polarimetrically by the molybdate method. Isocitric acid concentrations calculated from polarimetric readings by previous investigators who were unaware of the citrate effect require revision.

3. Preliminary experiments show that the equilibrium mixture of isocitrate, *cis*-aconitate and citrate in the presence of liver or muscle aconitase contains 6·2% isocitrate, 4·3% *cis*-aconitate and 89·5% citrate (38°; *p*H 6·8; 0·025*M* phosphate buffer). The effect of *p*H is small between 6·8 and 7·4. $MgCl_2$ shifts the equilibrium in favour of citrate.

4. A modified polarimetric method is suggested for the determination of malic and isocitric acids, applicable to solutions containing citrate.

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Aerobic Oxidation of Aromatic Hydrocarbons in the Presence of Ascorbic Acid

THE REACTION WITH ANTHRACENE AND 3:4-BENZPYRENE

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It has long been known that oxidation of the aromatic hydrocarbons plays a fundamental part in their elimination from the animal body, but isolation of the products excreted has contributed little to knowledge of the initial introduction of oxygen into the molecule. This problem has assumed even greater importance recently in connexion with the fate of carcinogenic hydrocarbons in the animal body. Many such hydrocarbons are now known and, in most cases, introduction of oxygen into the molecule leads to considerable or complete loss of carcinogenic activity. Thus in these carcinogens oxidation is equivalent to 'detoxication' from the point of view of cancer induction.

It is well known that the feeding of naphthalene to rabbits leads to the production of cataract. The crystalline lens normally contains much ascorbic acid. Simultaneously with the development of opacity in the lens during cataract formation the ascorbic acid content falls to a very low value. It has also been reported that the administration of ascorbic acid to animals on a naphthalene diet inhibits cataract production. It must be admitted that the whole question of the relation of ascorbic acid to cataract production by naphthalene is still undecided, and there is no general agreement on the facts. However, these experiments suggested to Jorissen [1937] the desirability of testing the

action of ascorbic acid on naphthalene *in vitro*. In the aqueous acetone solution of naphthalene containing ascorbic acid, he detected the formation of an oxygen-containing derivative of naphthalene which he assumed to be a naphthol.

This observation has been studied in more detail in the present work and extended to other hydrocarbons. Evidence of oxidation of the hydrocarbon has been obtained with naphthalene, phenanthrene, anthracene, and with the carcinogenic hydrocarbons 3:4-benzpyrene, cholanthrene, and methylcholanthrene. Experiments on the identification of the products from anthracene and 3:4-benzpyrene are described.

EXPERIMENTAL

The technique was very simple. The hydrocarbon was dissolved in a suitable volume of 80 % acetone (B.D.H. Analar) and 2–4 times its weight of ascorbic acid added. The flasks, which were large enough to contain ample air, were stoppered and kept in the dark at room temperature (usually about 20°). Light was excluded to avoid photo-oxidation.

Anthracene

Examined after 2 weeks, solutions containing anthracene and ascorbic acid showed no obvious difference in colour when compared with similar control solutions containing no ascorbic acid. Water was added to the solution and the acetone removed under reduced pressure at a low temperature. The solid material which separated (mainly unchanged anthracene) was collected and well washed with water. This residue was heated with *N* NaOH and Zn dust. The filtrate from Zn and unchanged hydrocarbon was deep red. The extraction with alkali and Zn was repeated until the filtrates and washings were colourless. On aeration of the alkaline filtrate a brownish solid separated and the solution lost its deep red colour. The solution was made slightly acid and the precipitate collected and dried. Recrystallization from glacial acetic acid gave fine yellow needles, M.P. 279–280° (uncorr.), which did not depress the M.P. (279–280°) of authentic anthraquinone.

In solutions containing anthracene and ascorbic acid left for longer periods (2–3 months) crystals of anthraquinone were seen to form as long very pale yellow needles, which on removal and recrystallization had M.P. 279–280°. No anthraquinone could be detected in the control experiments.

3:4-Benzpyrene

This hydrocarbon invariably showed a most striking and characteristic colour change. Benzpyrene in aqueous acetone kept in the dark maintains its pale yellow-green colour even after 5 years. In the presence of ascorbic acid the solution rapidly becomes deep yellow, then orange and finally ruby red. The change is detectable in a few hours, and after standing overnight it is quite obvious.

Isolation of benzpyrene quinones. After removal of the acetone under reduced pressure the precipitated solid was collected by filtration. This solid consisted mainly of unchanged hydrocarbon impregnated with some coloured material which gave the mass a magenta colour. Treatment of this product with 2*N* NaOH in the cold gave a dark alkaline solution with a strong green fluorescence. Acidification of this solution led to its decolorization with precipitation of a small quantity of red flocculent material. The nature of this material is discussed later.

The oxidation product was repeatedly leached with hot 2*N* NaOH until the extracts had lost all the green fluorescence and were practically colourless. Boiling the extracted residue with more 2*N* NaOH and Zn dust led to a red-brown vat. Unchanged hydrocarbon was removed by filtration and the alkaline solution aerated. A red precipitate separated and the solution became very pale green. After acidification the precipitate was collected and dried, when it was obtained as a brick-red powder. The yield was low—*ca.* 10 mg. from 100 mg. of benzpyrene. The melting-point after recrystallization from xylene was 230°, not depressed by admixture with a specimen of 3:4-benzpyrene quinone M.P. 230° [Cook & Hewett, 1933].

Vollmann, Becker, Corell & Streeck [1937] showed that the quinone described by Cook & Hewett is a mixture of 5:8- and 5:10-quinone. Separation was achieved by conversion of the mixture of quinones to the leucodiacetates and fractional crystallization. The 5:10-quinone crystallizes in golden orange needles M.P. 295° and dissolves in concentrated H_2SO_4 to give a carmine red solution. The 5:8-quinone forms red needles M.P. 245° and its solution in conc. H_2SO_4 is olive brown.

The product from ascorbic acid oxidation of benzpyrene gave a solution in conc. H_2SO_4 intermediate in colour between solutions of the pure quinones and indistinguishable from that of a solution of the mixed quinones of Cook & Hewett.

Other products of the oxidation. As mentioned above, some material is extractable from the oxidation product by alkali alone. Since this material can be apparently completely removed before the quinones are extracted by vat formation, it is not quinonoid but presumably phenolic in nature. On precipitation with acid from its alkaline solution it is red and flocculent, but on collection and drying it darkens. All efforts at purification of this material have so far been unsuccessful and have yielded nothing but further small quantities of quinone.

Colorimetric measurements on the oxidation product. These were made by means of a Hilger Photoelectric Absorptiometer using Ilford 'Spectrum' filters. The colour developed in mixtures of benzpyrene and ascorbic acid solution was measured and analysed by using the filters at intervals during 58 days. A typical result is shown in Fig. 1, in

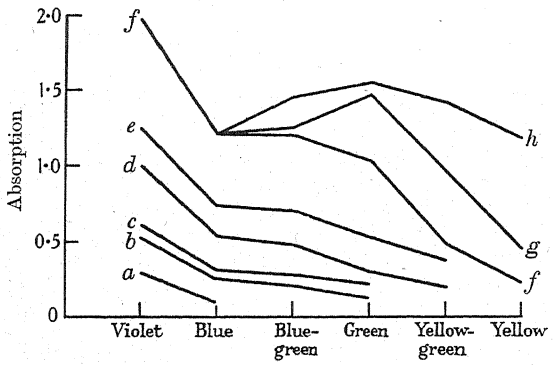


Fig. 1. Colour development in a solution of 3:4-benzpyrene during aerobic oxidation in the presence of ascorbic acid. $a=27$, $b=50$, $c=74$, $d=98$, $e=144$, $f=214$, $g=339$ hr. $h=58$ days.

which are plotted the values obtained by measurement of a solution initially containing 50 mg. benzpyrene and 200 mg. ascorbic acid. Similar measurements were carried out on pure specimens of the 5:8- and 5:10-quinone and on the mixture prepared by chromic acid oxidation of the hydrocarbon (Table 1). Attention may be directed to has fallen by 20 %. This change is not shown by the 5:10-quinone where the absorptions of saturated solutions in pure acetone or in 80 % aqueous acetone are practically indistinguishable.

Comparison of the values shown in the table with those for the ascorbic acid-hydrocarbon experiment given in Fig. 1 indicate that the colour developed in the latter is mainly due to formation of the quinones but that there is also some other chromogen present. In the later curves in the figure the absorption towards the red end of the spectrum is greater than can be accounted for by assuming a saturated solution of the mixed quinones, even if the more chromogenic 5:8-compound predominated.

The influence of various factors on the oxidation. By making use of the rapid development of colour with benzpyrene it was possible to examine the influence of various factors on the speed of the reaction. At temperatures of 30–40° the reaction proceeded more rapidly than at 20–25°, but measurements of the colour produced showed that the reaction at the lower temperatures, although initially slower, finally developed more colour. This is to be expected since the rate of destruction of the ascorbic acid is much greater at the higher temperature range. The rate of reaction was not appreciably increased by vigorous shaking in air or O_2, but the reaction is completely inhibited if O_2 is excluded. Mixtures of ascorbic acid and benzpyrene in aqueous acetone in sealed tubes from which all air has been removed or replaced by N_2 remain colourless for months if they are not exposed to

Table 1. *Absorption measurements on quinones*

Filter ...	V.	B.	B.-G.	G.	Y.-G.	Y.	O.	R.
5:10-Quinone (100 % acetone)	∞	1·07	0·47	0·12	—	—	—	—
5:10-Quinone (80 % acetone)	∞	1·03	0·47	0·11	—	—	—	—
5:10-Quinone (80 % acetone) + ascorbic acid	∞	1·20	1·10	0·65	0·20	—	—	—
5:8-Quinone (100 % acetone)	∞	1·22	1·22	1·06	0·17	—	—	—
5:8-Quinone (80 % acetone)	∞	1·18	1·20	1·50	0·48	—	—	—
5:8-Quinone (80 % acetone) + ascorbic acid	∞	1·23	1·28	1·55	1·15	0·53	0·185	0·06
Mixed quinone in 80 % acetone	∞	1·28	1·28	1·05	0·30	—	—	—

The values = absorption coefficient. Cell depth, 1·0 cm. Concentration—saturated at 15° C.

two points. One is the increase in intensity of the colour of the quinone solutions, particularly towards the red end of the spectrum, when ascorbic acid is present in their solutions. This is especially marked in the case of the 5:8-quinone where the absorption, for example, in the yellow-green is increased from 0·48 to 1·15 on adding ascorbic acid and allowing to stand for several days. The second point is that on diluting the 100 % acetone solution of the 5:8-quinone with water in order to obtain an 80 % solution there is an increase in the absorption intensity although the concentration of the quinone

bright light. On opening the tubes the usual sequence of colour changes takes place.

Since it appeared probable that the autoxidation of the ascorbic acid played an essential part in the mechanism of the oxidation, the effect of copper, cyanide, metaphosphoric acid, and H_2O_2 on the reaction was tried.

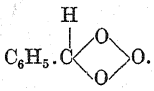
Small traces of cupric ions (1–2 μg./ml.) either had no effect or, occasionally, produced a slight acceleration of the reaction. M/100 KCN or 5 % HPO_3, however, completely inhibited the reaction. In view of the possibility that the oxidation of the

hydrocarbon might be due to H_2O_2 formed during autoxidation of the ascorbic acid, experiments were carried out in which H_2O_2 was added to the hydrocarbon-ascorbic acid mixture, and also some in which H_2O_2 alone without ascorbic acid was employed. The addition of the peroxide was without effect on the reaction when both it and ascorbic acid were present in the solution. Alone, the H_2O_2 did not oxidize the hydrocarbon.

These results indicated that the enediol grouping of ascorbic acid was the essential structural requirement for bringing about the oxidation of the hydrocarbon. Support for this view was obtained in experiments in which ascorbic acid was replaced by dihydroxymaleic acid. Exactly similar oxidations of hydrocarbons were obtained by the use of this acid in aqueous acetone solution.

DISCUSSION

The oxidation of aromatic hydrocarbons in the presence of substances undergoing autoxidation is well known. In a study of the inhibitory action of anthracene on the autoxidation of benzaldehyde Bäckström & Beatty [1931] have shown that the inhibitory action is connected with an induced oxidation of the inhibitor. The primary oxidation product is anthranol which is autoxidizable and reacts with oxygen to give a peroxide which is slowly decomposed with the formation of the final reaction product, anthraquinone. The course of the reaction indicated that the initial oxidation of the hydrocarbon is the result of a reaction with a peroxide of benzaldehyde of the structure



Wasley & Rusch [1942] showed that the autoxidation of benzaldehyde and of heptaldehyde was inhibited by anthracene, 3:4-benzpyrene, 1:2:5:6-dibenzanthracene, and 20-methylcholanthrene. In the case of benzpyrene, measurements of absorption spectra indicated that the inhibition involved the oxidation of the hydrocarbon to one or more quinones.

It seems probable that a similar mechanism underlies the reaction described in the present paper. The autoxidation of ascorbic acid may be assumed to involve the formation of a peroxide. If this peroxide oxidizes anthracene to anthranol and 3:4-benzpyrene to 5-hydroxy-3:4-benzpyrene as the initial products, the formation of anthraquinone and a mixture of benzpyrene quinones together with some monohydroxybenzpyrene is readily understood.

Whether such a mechanism plays any part in the *in vivo* oxidation and elimination of aromatic hydrocarbons remains undecided. Experiments on this point, and investigations of the possibility that aromatic hydrocarbons interfere with the normal utilization of ascorbic acid in the animal body, are in progress.

SUMMARY

Oxygen oxidizes aromatic hydrocarbons in aqueous acetone solutions containing ascorbic acid. Anthracene is oxidized to 9:10-anthraquinone and 3:4-benzpyrene yields a mixture of 3:4-benzpyrene-5:8-quinone and 3:4-benzpyrene-5:10-quinone. The oxidizing agent appears to be one of the products formed during autoxidation of ascorbic acid and is possibly dehydroascorbic acid or a peroxide thereof. The mechanism seems to be similar to that which occurs during the inhibition of the autoxidation of aldehydes by aromatic hydrocarbons.

I should like to express my thanks to the Sir Halley Stewart Trust for a Fellowship held during the course of this work. The British Empire Cancer Campaign, in addition to generous support of this Institute, put at my disposal a supply of 3:4-benzpyrene used in this investigation.

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The Action on Avian Malaria of the Alkaloids of Cinchonas from the Cameroons and the Belgian Congo

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During the course of the war it has become increasingly important to seek efficient and cheap remedies against malaria among the alkaloids of the cinchonas.

Numerous experiments on cinchona febrifuges have been carried out in several countries, e.g. Ejercito & Santos [1937], Field [1934], Fourneau, Trefouel, Trefouel, Stefanopoulo, Benoit, Lestrange & Melville [1930], Hicks & Chand Diwan [1935], Kingsbury [1937], League of Nations [1934; 1937], Manon, Perez & Russell [1935], Miyahara [1938], Parvulescu & Boeriu [1934], Sergent, Sergent & Vogt [1925], Sinton [1929], Slatineanu, Ciuca, Balteanu, Alexa, Francke & Rugina [1934], Wijerana [1939], Yao & Yung [1934]. From all these experiments it appears that in the treatment of ordinary cases of malaria cinchona febrifuges are equal to, if not of slightly superior value to, quinine. Nevertheless these cinchona febrifuges vary largely in composition, in origin and in the mode of preparation, and it seems desirable at the present time to obtain more detailed and accurate information about the nature of the total alkaloids from different cinchonas, and to examine their therapeutic action, before considering their intensive cultivation and the establishment of small plants for the purpose of extracting these alkaloids on the spot by simple processes.

The initial experiments to be described are confined to the action on avian malaria of alkaloids from the barks of *C. succirubra* and *C. Ledgeriana* from Dschang (French Cameroons) and Kivu (Belgian Congo). Some experiments on avian malaria have already been carried out by several authors: Bishop [1942]; Buttle, Henry & Trevan [1934]; Buttle, Henry, Solomon, Trevan & Gibbs [1938]; Giemsa [1933]; Goodson, Henry & Macfie [1930]; Sergent, Sergent & Vogt [1923; 1924; 1925]. The alkaloids used in the present study were either extracted by the classical laboratory method or by industrial processes. The samples were in all cases prepared in our laboratories and were practically pure (95–97 %), containing the same percentage of quinine as the laboratory product. Some small differences may exist in the relative proportions of the amorphous alkaloids present.

EXPERIMENTAL

The malaria parasites used in this work were a strain of *Plasmodium relictum* obtained from Dr Keilin of Cambridge to whom we wish to offer our thanks. The birds used were all canaries; owing to difficulties in obtaining supplies these were not all of a constant strain, but gave a uniform response to the infection.

The malaria was transmitted by direct blood inoculation from bird to bird. Blood was obtained from the vein running down the inside of the leg of an acutely infected bird and was collected in an equal volume of citrate-saline (NaCl 0·8 %, Na citrate 2 %). Each bird to be inoculated received 0·25 ml. diluted blood, injected into the pectoral muscle. Usually the examination of the blood of infected birds was begun on the fifth day, the blood films being treated with Giemsa's stain. At least 30 fields were examined on each slide.

The alkaloids were administered orally to the birds in the form of small pellets, which were prepared by mixing 1 part of alkaloid with 9 parts of corn flour, together with a little H_2O. Each pellet thus contained the amount of alkaloid (in form of insoluble base) required for a test on a single bird. The pellets were readily swallowed, and experience has shown that this method was more convenient than administration by catheter [Fourneau *et al.*, 1930].

The routine method of testing compounds was as follows: 6 hr. after a bird had been inoculated, a dose of alkaloid was given and five further doses were given at 24-hr. intervals. There were two untreated control birds in each experiment.

The birds chosen for each test were of approximately equal weight (to within 2 g.) and uniform doses were administered. It was found that under these conditions, the appearance of parasites in the blood is not dependent on the weight of the bird, either in untreated controls or in treated birds.

Most of the experiments were carried out with a daily dose of 3·5 mg. of total alkaloid, an amount which gave a good therapeutic effect without toxic symptoms.

RESULTS

The therapeutic effect of the alkaloids is measured in terms of the retardation of infection in the different groups. The results of experiments are summarized in Tables 1 and 2. In only one bird of the whole series (30 mg. of alkaloid from *C. succirubra*) was the onset of malaria indefinitely retarded.

The alkaloids from *C. Ledgeriana* in doses of 2·5 and 3·5 mg. were more effective than the same amount of quinine.

In all the experiments no differences were observed between the industrial and laboratory preparations from the different sources.

(ii) *Total alkaloids minus quinine.* Three of the

Table 1. *Influence of alkaloids* per os *on the time of onset of avian malaria*

Alkaloid	Source of alkaloid: Country	Source of alkaloid: Bark	Method of extraction	Influence of alkaloid on onset of malaria in canaries: Daily dose given (mg.)	No. of birds	Period of delay in onset of malaria (days)
Quinine (base)	—	—	—	2·5	4	5·25
				3·5	6	9·40
				5·0	4	11·00
Total	Cameroons	*C. succirubra*	Laboratory	2·5	4	4·25
				3·5	9	7·35
				5·0	4	>15·20
Total	Cameroons	*C. succirubra*	Industrial	2·5	3	3·70
				3·5	2	7·50
Total	Cameroons	*C. Ledgeriana*	Laboratory	3·5	5	10·20
Total	Cameroons	*C. Ledgeriana*	Industrial	2·5	3	8·00
Total (Sample R)	Belgian Congo	*C. succirubra*	Laboratory	2·5	4	8·50
Total (Sample M)	Belgian Congo	*C. succirubra*	Industrial	2·5	4	9·00
Total minus quinine (Sample E)	Belgian Congo	*C. Ledgeriana*	Industrial	2·5	2	4·50
Total minus quinine	Cameroons	*C. succirubra*	Industrial	2·5	1	4·50
Total minus quinine	Cameroons	*C. Ledgeriana*	Industrial	2·5	2	4·00

Table 2. *Composition of the total alkaloids tested*

Source and method of extraction	Total alkaloid in barks (%)	Composition (%) of total alkaloid: Quinine base	Cinchonidine	Cinchonine	Amorphous alkaloids	Impurities
C. succirubra (Dschang). Industrial	6·40	33	27	14	26	3
C. Ledgeriana (Dschang). Industrial	7·50	62	—	—	—	1
C. succirubra; sample R (Kivu). Laboratory	8·35	22	—	—	—	—
C. succirubra; sample M (Kivu). Industrial	7·00	13	—	—	—	5
C. Ledgeriana; sample E (Kivu). Industrial	7·25	59	—	—	—	—

DISCUSSION

(i) *Total alkaloids (including quinine).* The results indicate that the total alkaloids from all the sources examined have an effect similar to that of quinine in retarding the onset of avian malaria. The effect appears to vary largely according to the origin of the barks—the Belgian *C. succirubra* (M and R) gave a mixture of alkaloids which, although less rich in quinine, seemed to be more effective than the mixture extracted from the French bark.

The alkaloids of *C. succirubra*, when given in doses of 3·5 mg./day, were somewhat less effective than the same amount of quinine base, but on increasing the dose to 5 mg./day, these alkaloids (from the Cameroons) exerted a more powerful therapeutic action than quinine.

industrial preparations have been tested after the removal of their contained quinine. In a dosage of 3·5 mg./day they produced a retardation of avian malaria for 4–5 days, compared with the figure 7·35–10·5 days observed with the similar dose of alkaloids including quinine. The explanation of this difference may lie in a synergistic action of small amounts of quinine and other alkaloids from cinchonas, and experiments are in progress to elucidate this very important question.

SUMMARY

1. The total alkaloids from *Cinchona succirubra* and *C. Ledgeriana* from both the Cameroons and the Belgian Congo, have been tested for therapeutic action on avian malaria.

2. In all cases the results indicate that the total alkaloids have an effect similar to, or even better than, that of quinine.

3. Industrial and laboratory preparations are of equal value.

4. The total alkaloids from which the quinine has been extracted have a poor therapeutic effect, and the difference between these and the results with the total alkaloids may be due to the synergistic action of small amounts of quinine and other alkaloids.

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The Reducing Properties of Fibrinogen

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The protein, fibrinogen, is characterized by (1) its low solubility in moderately strong salt solutions, (2) its conversion to fibrin by thrombin and (3) its marked instability and ease of denaturation. The last property has made difficult its purification and study but it is now possible [Florkin, 1930] to prepare fibrinogen free from other proteins and without any evidence of denaturation. Study of the clotting of this purified fibrinogen by thrombin [Jaques, 1938] could not be continued until a method was found to stop the reaction quickly in order to obtain samples for analysis. The methods commonly used in enzyme chemistry for this purpose (heat, etc.) are inapplicable because they affect the solubilities of fibrinogen and fibrin. Dilute iodine, potassium permanganate and potassium dichromate, tested for this purpose, were found to give a heavy precipitate with fibrinogen. As this phenomenon was not found with the serum proteins and does not appear to have been reported before, it was investigated. Secondary reactions took place with $K_2Cr_2O_7$ and $KMnO_4$, as shown by re-solution of the precipitate. The study was therefore limited to the action of iodine and H_2O_2.

EXPERIMENTAL

Materials

Fibrinogen. Preparation by repeated precipitation with concentrated salt solutions is not very satisfactory in practice. The principle of Florkin [1930], who found it necessary for preparation of homogeneous solutions of the protein that all operations should be made at 2° and at *p*H 6·0–7·0, has been followed. Fibrinogen is rapidly denatured by acid even at low temperatures [Fay & Hendrix, 1931].

Florkin used NaCl as the precipitant, but the resulting 'salt of fibrinogen' tends to form gels and it is difficult to

control the rates of stirring and of addition of the salt, so as to obtain precipitates which dissolve readily. Also, the NaCl used must be carefully decalcified or the fibrinogen will clot after the first precipitation. Fibrinogen precipitated with ammonium and potassium sulphates or phosphates flocculates and has not the same tendency to form gels [cf. Ferguson & Erickson, 1939]. Instead of $(NH_4)_2SO_4$ [Ferguson & Erickson, 1939], phosphate buffer has the advantages that the *p*H is exactly controlled at all stages and the protein content of the solutions can be easily determined by nitrogen analyses. Butler & Montgomery [1932; 1935] have drawn attention to the particularly valuable properties of the equimolecular mixture of K_2HPO_4 and KH_2PO_4, *p*H 6·6, used by Florkin and other associates of Cohn. The method of Florkin has been found by the author to be facilitated if this phosphate buffer is substituted for NaCl. The 3 *M* phosphate buffer of Butler & Montgomery is diluted to 2 *M* before use, and the fibrinogen is precipitated by adding 1 vol. of this solution to 1 vol. plasma. The concentration of phosphate (*M*) appears to correspond to $\frac{1}{2}$ sat. NaCl solution in its precipitating power. The fibrinogen is washed with *M* phosphate buffer and redissolved in a vol. of *M*/4 phosphate buffer slightly less than that of the original plasma. Three such successive precipitations are made. All operations are conducted in the cold room at 2°. All solutions and glassware are chilled before use and it is advisable to rinse the glassware with a weak solution of Na citrate. The salt solution is added slowly to the continuously stirred solution exactly as directed by Florkin. The use of a centrifuge or a Sharples Super Centrifuge installed in the cold room will be found much preferable to filtration. With these modifications, the whole preparation can be readily carried out in 24 hr. The fibrinogen used in this study was prepared from citrated horse plasma, kindly supplied by Dr E. G. Kerslake of the Connaught Laboratories, University of Toronto.

A simple test to demonstrate the complete absence of other plasma proteins in the fibrinogen solution is the lack of any antithrombin action of heparin when tested with the fibrinogen [Jaques & Mustard, 1940]. Mere traces of plasma are sufficient to restore the antithrombin action of heparin.

Methods

The fibrinogen was dissolved in *M*/4 K phosphate buffer (*p*H 6·6). The reaction vessels were 25 ml. glass-stoppered Erlenmeyer flasks rocked in a thermostat at 25°. 1 ml. fibrinogen solution (0·5 mg. N) + 4 ml. of H_2O were taken and 1 ml. *N*/75 I_2 in KI or H_2O_2 added. The consumption of I_2 by the blank was very small but a control with phosphate buffer instead of the fibrinogen solution was always made. The value reported for the I_2 or H_2O_2 consumption represents the difference between the titre of the control and the titre with fibrinogen added.

The reaction was stopped by adding 1 ml. of 60% KI. The residual I_2 was then titrated with standard *N*/200 $Na_2S_2O_3$ using a 2 ml. microburette and starch as an internal indicator. Residual H_2O_2 was estimated by titrating the equivalent I_2 liberated from the KI. For this, 1 ml. of 4*N* H_2SO_4 was added at the same time as the KI, and after 5 min. the contents were titrated. Clotting was carried out as by Jaques [1938]. The thrombin had been purified by the methods of Seegers and was kindly supplied by Parke, Davis & Co., Detroit.

RESULTS AND DISCUSSION

Kinetics of the oxidation of fibrinogen by H_2O_2 or I_2

The value of *K*, the velocity constant of a bimolecular reaction, was calculated from the data of Table 1 by the equation:

$$K = \frac{1}{t\,(C_A - C_B)} \log_e \frac{(C_A - C_x).C_B}{(C_B - C_x).C_A}.$$

The values of *K* for the reaction with H_2O_2 show very good agreement. This indicates that the reaction is bimolecular and shows that probably a specific reacting group is involved. The values with I_2 do not show such good agreement. This was

Table 1. *Reaction of fibrinogen with H_2O_2 and I_2*

H_2O_2: 1 ml. *N*/100 H_2O_2 + 2 ml. fibrinogen (0·890 mg. N)			I_2: 1 ml. *N*/75 I_2 + 1 ml. fibrinogen (0·500 mg. N)		
Reaction time hr.	H_2O_2 consumed ml.	*K*	Reaction time min.	I_2 consumed ml.	*K*
0·5	0·164	0·00430	8	0·511	0·0179
1·0	0·263	0·00398	25	1·044	0·0179
2·0	0·443	0·00428	48	1·250	0·0141
3·0	0·535	0·00414	75	1·381	0·0153
5·0	0·629	0·00378	79	1·435	0·0132
6·6	0·762	—	120	1·562	0·0126
8·0	0·763	—	300	1·713	—
22·0	0·763	—	720	1·711	—
C_A = 1·953 C_B = 0·763			C_A = 2·750 C_B = 1·712		
K (average) = 0·00410			*K* (average) = 0·0152		

(All concentrations are expressed as equivalent ml. of *N*/200 $Na_2S_2O_3$.)

partly due to technical difficulties but, as indicated later, the reaction with I_2 probably involves several groups. The results with I_2 indicate, however, that here also the reaction involves specific groups of the protein. The velocity of the reaction is fairly high, e.g. with I_2 equilibrium was reached in 5 hr. This was in the presence of 0·06% KI from the standard I_2 solution, and KI strongly inhibits the reaction. At equilibrium, 2·17 mg. I_2 and 0·146 mg. H_2O_2 were reduced per mg. fibrinogen. The same equilibrium values were obtained when the relative concentrations of fibrinogen and oxidizing agent were varied. Iodine evidently takes the oxidation further than H_2O_2: the I_2 consumed is just 4 times the H_2O_2—at equilibrium 3·42 ml. *N*/200 I_2 compared with 0·856 ml. *N*/200 H_2O_2/mg. N.

The effect of various factors on the reaction is shown in Fig. 1: (*a*) varying amounts of KI, followed by 1 ml. *N*/75 I_2, were added to a series of tubes and the tubes allowed to stand 7 hr. KI strongly suppresses the reaction, a final concentration of 6% being sufficient to inhibit it completely.

(*b*) Fig. 1*b* shows the effect of *p*H. The amount of I_2 reduced in 1 hr., and of H_2O_2 in 5 hr., was determined. The *p*H level was established by adding before the oxidizing agent either $N/2$ H_3PO_4 or $N/2$ KOH to the fibrinogen solution. From zero reduction at *p*H 2·3, the amount of I_2 reduced rose rapidly with increasing *p*H. Beyond *p*H 7·5 the 'blank' consumption of I_2 became so great that it interfered with the titrations, but there was no evidence of a decreased consumption by fibrinogen. The effect of *p*H on the reduction of H_2O_2 is similar to but less than the effect on I_2. There appears to be a definite break in the *p*H-curve at the isoelectric point of fibrinogen, the H_2O_2 consumption increasing only relatively slowly on its alkaline side. Isoelectric precipitation of fibrinogen occurred from *p*H 4·4 to 5·4. With I_2, precipitation occurred from *p*H 3·1 to 7·5. With H_2O_2, precipitation was observed only in the region of the isoelectric point of fibrinogen. (*c*) Fig. 1*c* shows the I_2 consumption found with varying amounts of fibrinogen, the phosphate concentration being always $M/12$. The I_2 consumption was determined after 1 hr. The resulting curve approximates to a straight line. The approximately linear relationship and the close agreement observed with duplicates (Fig. 1*c*) suggest that the reaction might be used for the determination of fibrinogen. When the oxidation proceeded for only ½ hr., the graph tended to become parabolic.

The results obtained with I_2 (inhibition by KI, rapid increase in the amount of I_2 reduced, with rise in *p*H, etc.) closely resemble those obtained in the iodimetric determination of cysteine and related compounds [Lucas & King, 1932] and suggest that sulphydryl group is responsible. Fibrinogen does not contain a free SH group, however, for it gives a negative nitroprusside reaction. Very little iodine substitution has occurred in the protein, as shown by analysis. It appears likely, therefore, that the reduction of I_2 and H_2O_2 by fibrinogen is due to a group other than sulphydryl.

The reaction of the serum proteins with I_2

A qualitative difference between the reactions with serum proteins and with fibrinogen is evident from the fact that the former do not give any precipitate with I_2. The I_2 consumption of a sample of horse plasma was therefore determined as usual and was corrected for the reducing power of the fibrinogen present. 0·10 ml. plasma (1·10 mg. N) gave an I_2 uptake for 1 hr. of 1·964 ml. Total fibrinogen N of the sample was found to be 0·08 mg. equivalent to 0·211 ml. Therefore the I_2 uptake of the serum proteins = 1·72 ml./mg. N, which is a little lower than the value for fibrinogen (2·64 ml./mg. N).

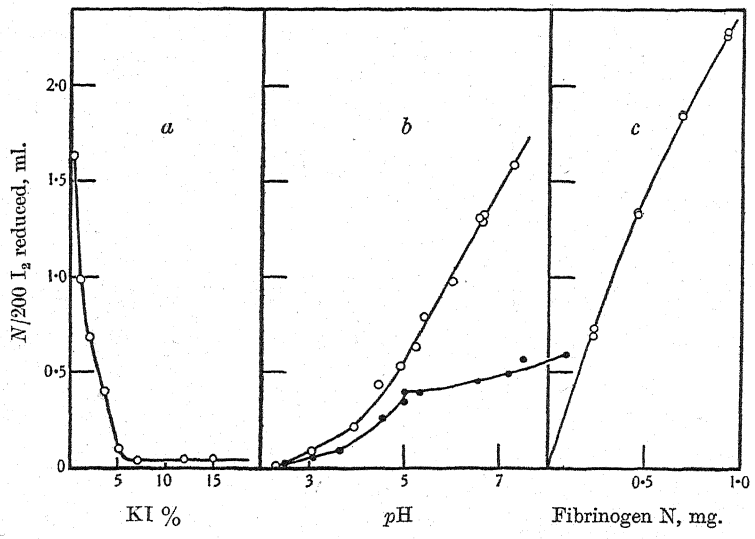

Fig. 1. Effect of various factors on the reaction: *a*, effect of potassium iodide; *b*, effect of *p*H; *c*, effect of fibrinogen concentration. o—o $N/200$ I_2 reduced; •—• $N/200$ H_2O_2 reduced. Fibrinogen present, as mg. N: *a*, 0·500; *b* (I_2), 0·425, *b* (H_2O_2), 0·850. Iodine or peroxide present, 1 ml. of *ca.* $N/100$. Total volume, 6 ml., and phosphate concentration $M/24$, in all.

Effect of denaturation on the reducing properties of fibrinogen

Mirsky & Anson [1936] found that denaturation of proteins increased their reduction of ferricyanide. This was not found with the I_2 reduction by fibrinogen (Table 2). The fibrinogen was denatured in a water-bath at 56°, when an increase in the reducing

Table 2

A. *Effect of denaturation on the reducing properties of fibrinogen*

Incubated at 56° min.	Insol. N mg.	$N/200$ I_2 reduced ml.	$N/200$ H_2O_2 reduced ml.
0	0·000	1·167	0·372
0·5	0·022	1·174	—
2	0·252	1·195	0·366
5	0·325	1·194	0·355
10	—	1·143	0·322
36	0·322	1·094	—
60	—	1·038	0·200
240	—	0·847	—

(H_2O_2 consumption for 5 hr. and for 2 ml. fibrinogen solution (1·0 mg. fibrinogen N). I_2 uptake determined at 25° for 1 hr. for 1 ml. fibrinogen solution (0·5 mg. fibrinogen N).)

B. *Effect of denaturation on the reducing properties of plasma*

Temp. ° C.	$N/200$ I_2 consumed ml.
25	0·955
40	0·956
60	0·957
80	1·113
100	1·179

(Plasma diluted 1 : 25 with H_2O and exposed to the raised temperature for 5 min. I_2 consumption in 1 hr. at 25° was determined for 1 ml. of the diluted plasma.)

value of not more than 2 % occurs in the first 5 min. After prolonged heating, the reducing value falls rapidly, but this was due to a decrease in the rate of reaction, since no difference between the denatured and native fibrinogen was found when the I_2 uptake at equilibrium (12 hr. oxidation) was determined. Similar results were obtained when H_2O_2 was used, but there was no evidence of any increase in the reducing value, which shows a gradual but definite decrease from that of native fibrinogen. In view of the unstable nature of fibrinogen, it was of interest to see if atmospheric oxygen had any effect on the reducing properties of the protein. No difference between the reducing properties of samples of fibrinogen kept in N_2 or in O_2 could be detected, even after prolonged incubation at 56°.

The effect of alkali and acid, to which fibrinogen is very sensitive, was tested on the reducing properties of the protein. Samples of the protein were kept 24 hr. at 25° at various *p*H levels, and their I_2 consumption at *p*H 6·6 was then determined. There was very little effect, however, so that this lability is not associated with or accompanied by a change in the reducing groups.

While denaturation has little effect on the reducing properties of fibrinogen, it definitely increases the reducing power of plasma, as shown in Table 2B. 3 ml. of water were added to 1 ml. of a 1 : 25 dilution of plasma and the tube was placed in a water-bath at the desired temperature for 5 min. The tube was then cooled, 1 ml. phosphate buffer and 1 ml. I_2 added, and the I_2 consumption after 1 hr. determined. There is no increase in the I_2 consumption after heating at temperatures below 80°, i.e. the denaturation of fibrinogen causes no increase in the I_2 consumption. On heating to 100°, where denaturation of the serum proteins will occur, there is a definite increase (20 %) in the I_2 consumption.

Changes in the reducing power of fibrinogen during clotting

To 5 ml. solution (0·880 mg. fibrinogen N in $M/10$ phosphate buffer) was added 0·1 ml. of a crude thrombin solution (0·021 mg. N). After 10 min., when a firm clot had formed (fibrin N = 0·564 mg.), 1 ml. $N/75$ I_2 was added and the amount of I_2 reduced after 1 hr. determined. For the clot this was 1·18 ml., while for the fibrinogen and thrombin it was 2·24 + 0·065 = 2·30 ml. This result indicates less reduction by the fibrin but does not distinguish a slower rate of reaction from a lower reducing power. Therefore, the amounts of H_2O_2 and I_2 reduced by the time equilibrium had been attained (30 hr.) were determined. To 5 ml. solution containing 1·0 mg. fibrinogen N was added 0·1 ml. thrombin solution (0·01 mg. Parke, Davis thrombin). After 3 hr. (fibrin N = 0·762 mg.) 1 ml. $N/50$ H_2O_2 was added. 0·903 ml. $N/200$ H_2O_2 was reduced after clotting, compared with 0·819 ml. for the fibrinogen control. This represents an increase of 0·110 ml./mg. of fibrin N, i.e. 13 %. In a similar experiment with I_2, the amount of I_2 reduced after clotting (fibrin N = 0·362 mg., original fibrinogen N = 0·478 mg.) was 1·818 ml., that of the fibrinogen control, 1·812 ml.

The reducing power of fibrin at equilibrium was more accurately determined with commercial beef fibrin (Table 3). 10 mg. samples were weighed out

Table 3. *The reduction of H_2O_2 and I_2 by fibrin*

Time hr.	$N/200$ H_2O_2 reduced ml.	Time hr.	$N/200$ I_2 reduced ml.
6	1·390	1·3	3·32
12	1·792	3	3·83
17	1·810	3·5	3·86
20	1·808	7·5	5·13
24	1·898	19	5·57
28	1·695	24	5·26
36	1·841	27	5·95
40	1·870	29	5·95

(10 mg. fibrin = 1·401 mg. N.)

on the micro-balance and 5 ml. $M/20$ phosphate buffer and 1 ml. H_2O_2 or I_2 solution added. The

average values for the amount of $N/200$ H_2O_2 and I_2 reduced at equilibrium were 1·823 and 5·57 ml. This gives values of 1·301 and 3·98 ml./mg. N. The previous values found for fibrinogen were 0·855 and 3·42 ml., so that the increase in reducing power is equivalent to 0·446 ml. H_2O_2 and 0·56 ml. I_2. The increase in reducing power is approximately the same with the two reagents but, owing to the more extensive oxidation by I_2, the increase with this reagent is a much smaller percentage of the total titration and hence the experimental error is increased accordingly. The increase is equivalent to half the reducing power of fibrinogen for H_2O_2.

These high concentrations of I_2 and H_2O_2 appear to have little effect on the activity of thrombin. Further, after oxidation of fibrinogen with H_2O_2, the addition of thrombin still results in a normal clot. The product resulting from the action of I_2 on the protein, however, is not clotted by thrombin, although thrombin still attacks it. The more extensive oxidation by I_2 evidently interferes with the gel properties of fibrin, since no clot is formed by the thrombin and the altered fibrin is merely precipitated.

The reduction of H_2O_2 by non-protein substances under the conditions tested

As a possible indication of the nature of the reducing groups of fibrinogen, 20 mg. of various substances were weighed into flasks, and 1 ml. of $M/4$ phosphate buffer and 4 ml. water added; 1 ml. of $N/75$ H_2O_2 was then added, the flask shaken for 28 hr., and the excess H_2O_2 titrated (Table 4).

Table 4. *Reduction of H_2O_2 by various substances*

Substance (20 mg. unless otherwise indicated)	$N/200$ H_2O reduced ml.
l-Tyrosine	0·000
l-Tryptophan	0·246
2-Methyl-1:4-naphthoquinone	0·161
2-Methyl-1:4-naphthohydroquinone	0·247
Glucose	0·603
p-Dimethylamino-benzaldehyde	0·950
Casein	0·726
Fibrinogen	2·402
Cysteine HCl (1·0 mg.)	0·822
Ascorbic acid (0·1 mg.)	0·451

Tyrosine did not reduce H_2O_2. Tryptophan, glucose and vitamin K showed slight activity but this was very small compared with that of fibrinogen. Cysteine was more active than fibrinogen, but on the basis of these reduction figures fibrinogen would contain 15% cysteine and fibrin 22%: values reported for the cysteine content of fibrin are from 1·5 to 3·7% [Jordan Lloyd & Shore, 1938]. This suggests that cysteine is not responsible for the reduction by fibrinogen, and it seems reasonable to assume that the reducing group will not be more than 5% of the protein. The high reducing power of the active group is shown by the fact that only ascorbic acid gave a reduction of this order.

DISCUSSION

The characteristic reaction of fibrinogen with iodine appears to indicate a further example of the non-sulphydryl reducing groups of proteins studied by Mirsky & Anson [1936] and more recently by Bowman [1941 *a*, *b*] and ascribed by them to the tyrosine and tryptophan residues of the protein. Mirsky & Anson studied the reduction of ferricyanide by edestin, serum globulin, egg albumin and gelatin. There appear to be marked differences between the reaction studied by them and that reported here, although the same group appears to be involved. They found that their reaction was not clearly defined, for even when the ferricyanide concentration was 30 and 50 times that reduced by the protein, the amount reduced still depended on the ferricyanide concentration. This is in marked contrast to the present results, which show a clearly defined bimolecular reaction. Also, they found little reduction of the ferricyanide unless the protein had been denatured. Fibrinogen reduces no more H_2O_2 or I_2 after denaturation than before, although denatured serum proteins do show an increase in reduction. Bowman observed the reduction of I_2 by the chorionic gonadotropic hormone, and found that the reaction was inhibited by KI, and that the presence of phosphate markedly and specifically accelerates the reaction. The fibrinogen used in the above experiments was dissolved in phosphate buffer. Bowman found that in the presence of phosphate, tyrosine reduces I_2 very rapidly at room temperature, the reaction being influenced by KI and *p*H in the same way as the reduction by protein. But tryptophan may be equally responsible for the reducing power, since under the same conditions tryptophan shows equally strong reducing properties.

The results with H_2O_2 indicate, however, that fibrinogen contains another reducing group with much stronger reducing properties than tyrosine or tryptophan. This group reduces H_2O_2 at *p*H 5–7. Further, there is an apparent increase in the number of such groups in the protein on conversion of the fibrinogen to fibrin. The nature of this group is unknown, cysteine and ascorbic acid being the only simple substances tested which show similar reducing power; but the negative nitroprusside reaction and the quantitative relationships found suggest that the former is not responsible.

SUMMARY

The action of H_2O_2 and I_2 on fibrinogen has been studied. Fibrinogen in phosphate buffer solution reduces these substances rapidly at 25° and *p*H 6·6. The reaction with I_2 is associated with the aromatic residues of the protein. The reducing power of fibrinogen is not changed by denaturation, but the reducing power towards H_2O_2 is increased on conversion to fibrin.

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The Vitamin B_1 Content of Potatoes

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The use of the mould *Phycomyces blakesleeanus* as a test organism for estimating vitamin B_1 was first worked out by Schopfer & Jung [1937*b*]. The fungus responds to such small quantities of the vitamin that the method is the most sensitive known; on this account it was adapted for estimating the vitamin B_1 content of human blood by A. P. Meiklejohn [1937] and Sinclair [1938; 1939]. The method has been used to estimate vitamin B_1 in plant materials by several American workers [Burkholder & McVeigh, 1940; Robbins, 1939; Bonner & Erickson, 1938]. It has the advantage, as compared with other methods, of requiring such small samples of plant material that it can be used to study the distribution of the vitamin in a single plant or part of a plant.

This paper deals with the use of the *Phycomyces* method for estimating the vitamin B_1 content of potato tubers, and with variations in the vitamin content and its distribution in the potato plant.

METHODS

The method employed was an adaptation, for use with plant material, of the method for blood estimations worked out by Meiklejohn and Sinclair. It consisted essentially in the comparison of the growth of *Phycomyces* in cultures containing potato samples with a dose-response curve obtained from a series of control cultures containing known amounts of the vitamin.

Medium. The *Phycomyces* cultures were grown in 50 ml. conical flasks; each flask contained 4 ml. of a mixture of equal parts of the following solutions in distilled water:

Glucose (A.R.)	Equal weights of solid and water
Asparagine (recrystallized)	4 g./100 ml.
KH_2PO_4 (A.R.)	1·5 g./100 ml.
$MgSO_4, 7H_2O$ (A.R.)	0·5 g./100 ml.

The volume of liquid in each flask was made up to 10 ml. by the addition of vitamin B_1 solution and distilled water, or distilled water only.

Control cultures. A range of 12 control cultures was set up in every experiment, containing the following amounts of vitamin B_1 in 10 ml. liquid: none, 0·05, 0·1*, 0·15, 0·2*, 0·25, 0·3* μg., and excess (2·5 or 5 μg.) (quantities marked * in duplicate). A solution of vitamin B_1 containing 1 μg./ml. was freshly prepared for each experiment from a stock solution in 75% ethanol (*p*H 4·5) kept in a refrigerator. The required volume of the dilute solution was added to medium and water in each flask, immediately before the adjustment of the *p*H.

Potato samples. The size of sample was designed to give a reading in the central part of the dose-response curve. Preliminary experiments showed that, for potato, samples of between 0·1 and 0·2 g. were correct. According to the amount of vitamin expected, samples were taken of 0·1, 0·15, or 0·2 g. (±10 mg.), in triplicate from each tuber, or part of a tuber, to be tested, by taking out cores with a small cork borer and cutting off the required length. Skin samples were taken by removing the corky skin and cutting a thin slice off the exposed surface, and sprout and leaf samples by cutting off a small piece. All samples were

quickly weighed and immediately blanched by putting them straight into the liquid medium in the culture flasks, which were held at a temperature of 80–100° in a steamer.

Sterilization. All culture flasks were sterilized by steaming for 45 min. on 3 successive days. Just before the second steaming, the dilute vitamin B_1 solution was added to the control flasks; some of the potato cultures received excess vitamin as well, and this was added at the same time, the *p*H of the liquid in all flasks being brought to 6·4 with *N*/20 NaOH. The potato samples were broken up with a glass rod.

Inoculation and incubation. Stock cultures of *P. blakesleeanus* (sex −) were maintained on dextrose-peptone-malt agar, and also on the experimental liquid medium, with potato and excess vitamin B_1. Inocula were made, normally from a liquid culture, by removing the aerial mycelium and stirring it in sterile distilled water. 1 ml. of the resulting spore suspension was pipetted into each culture flask. The flasks were incubated 12–16 days in the dark at room temperature.

Drying and weighing of fungus. At the end of incubation the mats of *Phycomyces* mycelium were removed from the culture flasks, washed 3 times in tap and once in distilled water, dried for 2–3 hr. at 100° and weighed to 0·1 mg.

Calculation of results

A dose-response curve was constructed from the 12 control cultures in every experiment by plotting the dry weight of mycelium in mg. (or the average of duplicates) against the logarithm of the amount of vitamin B_1 (μg.) in each culture. The curves were always **S**-shaped, and the middle part, corresponding to 0·1–0·25 μg. vitamin B_1, was very nearly a straight line [cf. Sinclair, 1939]. The amount of vitamin in the potato samples was calculated by reading from the curve the figure corresponding to the dry weight of fungus from each sample flask. The results are expressed as μg. vitamin B_1/100 g. fresh weight of potato.

RESULTS

A. *Comparison with thiochrome method.* The *Phycomyces* method is open to one serious theoretical objection; Schopfer & Jung [1937*a*] showed that the fungus responds to the pyrimidine and thiazole halves of vitamin B_1, if they are supplied together, as well as to the intact vitamin. It was therefore thought advisable to check the fungus method by comparing it with the thiochrome method as described by Pyke [1939]. The results of 3 comparisons on tubers of the variety Majestic are given in Table 1; the figures obtained by the 2 methods are of the same order in each case. This makes it reasonably certain that vitamin B_1 itself, and not its decomposition products, was being estimated by the fungus method.

B. *Variations during growing season.* In both 1941 and 1942, at intervals during the growing season, between July and late September, tubers were dug from plants in the same field and assayed for vitamin B_1. In 1941 the material was obtained from Spalding, Lincolnshire, by Mr P. H. Gregory; the two varieties Majestic and King Edward, growing in adjacent fields, were sampled by taking 1 tuber from each of 4 plants of each variety for each determination. In 1942 the material was obtained from a plot on the Rothamsted farm planted with Scottish seed Majestic; 2 tubers were taken from each of 2 plants at each sampling. Three cores were taken from the middle of each tuber.

From Table 2 it will be seen, first, that the samples are reasonably constant in vitamin B_1 content; the standard errors of the mean values for each sample are small enough to permit a valid comparison between samples.

Secondly, the vitamin B_1 content of the tubers of both varieties increases continuously during the growing season. The figures for Majestic show that the increase continues up to the time when main-crop potatoes are usually lifted, and also show a very good agreement between parallel samples in

Table 1. *Comparison of methods for estimation of vitamin B_1*

Date	Nature of material	Comparison	Vitamin B_1 (μg./100 g.) Fungus method	Vitamin B_1 (μg./100 g.) Thiochrome
April 1942	Stored tubers	Different tubers of same batch	76	61
			76	72
May 1942	Seed tubers (sprouted)	Different tubers of same batch	57	54
			46	—
			78	—
July 1942	Freshly dug young tuber	Both tests on same tuber	51	67

Table 2. *Variations in vitamin B_1 content of tubers dug in summer*

Date of digging	Majestic 1941 (μg./100 g.)	King Edward 1941 (μg./100 g.)	Date of digging	Majestic 1942 (μg./100 g.)
18. vii. 41	61 ±1·6	63·1 ±4·0	20. vii. 42	55·6 ± 4·7
9. viii. 41	74·8 ±3·9	67 ±2·1	12. viii. 42	75·6 ± 5·1
8. ix. 41	91·3 ±4·6	77·3 ±7·4	15. ix. 42	135·7 ±10·2
25. ix. 42	141·3 ±8·7			

Each figure is the mean of 12 determinations: 3 cores each from 4 tubers.

Table 3. *Effect of storage and sprouting on vitamin B_1 content of potatoes*

Date	Where stored	Variety	No. of tubers	Vitamin B_1 (μg./100 g.)	Variety	No. of tubers	Vitamin B_1 (μg./100 g.)
25. ix. 41	Crop as lifted	Majestic	4	141·3 ± 8·7	Doon Star	4	163 ±12·5
2. i. 42	Rothamsted	,,	3	162·2 ±13·0	—	—	—
	Sutton Bonington		3	138·2 ±12·3	—	—	—
5. ii. 42	Rothamsted	Majestic (sprouted)	4	112·4 ±22·2	King Edward (sprouted)	4	46·7 ± 7·8
8. iv. 42	Sutton Bonington	,,	4	99·9 ± 8·5	,,	4	73·7 ± 4·4
7. v. 42	Scottish seed fully sprouted	,,	3	61·8 ± 7·0	—	—	

1941 and 1942. King Edward tubers would appear to have a lower vitamin B_1 content than Majestic tubers.

C. *Effect of storage.* During the winter and spring of 1941–2, samples were taken from tubers stored in two places: at Rothamsted, where they were kept in paper bags on the laboratory floor, and at the Midland Agricultural College, Sutton Bonington, on trays in a well-ventilated storehouse. The cores for vitamin determinations (3 or 4) were again taken from the centre of each tuber (Table 3).

There certainly is a loss of vitamin B_1 from the centre of the tuber during storage, and apparently the greatest loss takes place in spring, after the tubers have begun to sprout. It may be conjectured that the greater part of the loss is due to sprouting, and not to storage *per se*. This conjecture is supported by the gradient in sprouting tubers (see Section D). Unfortunately there are not many results from stored material, and the material in some cases varied considerably in vitamin B_1 content. This is particularly so in the case of the Rothamsted-stored Majestic, as is shown by the high standard error.

D. *Distribution in the plants. Gradients.* It was thought possible that there might be a movement of vitamin B_1 into the rose end of a sprouting tuber, which would result in a gradient in vitamin content within the tuber. At different times of year, therefore, tubers were cut into 3 parts: the rose end, a middle slice (which is the part usually taken for sampling), and the heel end. Cores were taken from the 3 parts separately for determinations (Table 4).

Table 4. *Distribution of vitamin B_1 within single tubers*

Date	Variety	Condition of tuber	Position	Vitamin B_1 (μg./100 g.)
May	Majestic	Fully sprouted seed	Heel	38·4
			Middle	77·8
			Rose	67·7
Oct.	Majestic	Freshly dug, no sprouts	Heel	97·9
			Middle	101·7
			Rose	120·1
Oct.	Majestic	Freshly dug, no sprouts	Heel	117·8
			Middle	209·9
			Rose	165·7

There is consistently less vitamin in the heel end than in the rest of the tuber, but the evidence for a gradient cannot be regarded as satisfactory. There is, however, a very much higher content in sprouts than in the middle of the tuber, as is shown in Table 5. (These figures were obtained from white sprouts only, as green sprouts showed a curious anomaly, which is discussed in a later section.)

Table 5. *Sprouts compared with centre of tuber*

Date	Variety	Vitamin B_1 in sprouts (μg./100 g.)	Vitamin B_1 in tuber (μg./100 g.)
Jan.	Majestic	174	128
Feb.	Majestic	149	20
Feb.	King Edward	250	53

There was always less vitamin in the layer immediately under the corky skin than in the centre of the tuber; comparisons on different single tubers are given in Table 6. The skin layer, however, was always found to show adjuvant action; this is further discussed in a later section of this paper.

Table 6. *Skin compared with centre of tuber*

Date	Variety	Vitamin B_1 in centre (μg./100 g.)	Vitamin B_1 in skin layer (μg./100 g.)
Jan.	Majestic	179	22
Feb.	Majestic	206	9
		76	10
Feb.	King Edward	24	21
		49	18
May	Majestic	78	33

There is a very marked gradient of vitamin content in growing potato plants; in June 1942 two seed tubers of Majestic which had been planted a month previously were dug up and assayed together

with the small plants growing from them, which were then about 4 in. above the ground. Samples were taken from the heel, middle and rose ends of the seed tubers, the white stem below ground, and from the small leaves at the top of the stem. Sampling from 2 whole plants was repeated in July, when the tops were about 18 in. high. In this case samples were taken from the middle of 2 newly developed tubers, from an internode half-way up the stem, from the blades of a lower leaf, and from the terminal bud and the small leaves round it. The averages of 3 determinations at each point are given in Table 7.

Table 7. *Gradients in vitamin B_1 content in growing plants*

Date	Part of plant	Vitamin B_1 (μg./100 g.) Plant A	Plant B
8 June	Planted seed tuber: heel	25	27
	middle	39	24
	rose	47	40
	White stem below ground	103	93
	Foliage—small leaves	>1200	>1200
		Plant C	Plant D
20 July	Young tubers—middle	53	51
		78	41
	Green stem—internode	9	9
	Lower leaf—blade	129	153
	Terminal bud and leaves round it	>285	>115

In June the planted seed tubers have their highest vitamin B_1 content in the rose end; the stem has a rather larger content, and the value for the tops is enormously high. The amount of mycelial growth in the cultures on foliage was so large as to be right off the curve; the true vitamin content can only be guessed at. In July stems contain very little vitamin and leaves a good deal; but even the terminal buds do not approach the enormously high figure for June foliage.

E. *The presence in potato of adjuvant factors for the growth of* Phycomyces. *Phycomyces* responds to other growth factors than vitamin B_1; Schopfer & Jung [1937*b*] said that it requires 'une constellation de facteurs auxogènes.' Sinclair [1938] described the 'adjuvant action' of blood; blood and vitamin B_1 supplied to the fungus together produced more growth than the sum of the two separately. He attributes this action to a combination of causes, notably the buffering action exerted by blood, and its nitrogenous constituents.

In every experiment reported in this paper some cultures were grown on potato and excess vitamin, to test for the presence of adjuvant action; such action, shown by excess growth over the sum of potato and vitamin cultures separately, was exhibited by tuber material only in the cases shown in Table 8.

Table 8. *Potato tubers showing adjuvant action in vitamin B_1 test*

Date	Variety	Condition of tuber
1941		
June	Majestic	Fully sprouted seed
July	Majestic	Freshly dug, newly formed
Aug.	Majestic	Freshly dug, newly formed
1942		
Apr.	King Edward	From store, sprouted
June	Majestic	Seed, 1 month planted
July	Majestic	Freshly dug, newly formed
Aug.	Majestic	Freshly dug, newly formed

Mature tubers, from September to April, never displayed any adjuvant action, nor did seed potatoes at the time of planting in 1942. Samples of skin layer (see Table 6) always showed a marked action, but the factor was not present in sprouts. Stem from growing plants (Table 7) showed adjuvant action in 2 cases, but leaves never did.

The adjuvant factor in potato apparently operated only in presence of excess vitamin B_1, as is shown by the experiment reported in Table 9, in which parallel sets of cultures were set up, 1 with a range of concentrations of vitamin B_1 alone (column A), a 2nd with the same range of vitamin concentrations plus potato samples taken from a single tuber (column B), and a 3rd set containing samples from the same tuber without added vitamin (column C). The increased growth due to the addition of potato (B − A) is no larger than that produced by potato alone, except in the presence of excess vitamin (2·5 mg.).

Table 9. *Growth of fungus with potato alone and on potato plus vitamin B_1*

Vitamin B_1 supplied (μg.)	Fungus growth with vitamin B_1 only (mg.) A	Fungus growth with vitamin B_1 + potato (mg.) B	Diff. (B − A)	Growth with potato alone (mg.) C	Adjuvant action
0·05	18·2	51·7	33·5	36·8	None
0·1	31·2	72·2	41·0	42·0	None
0·2	57·2	87·8	30·6	39·2	None
0·3	69·2	103·2	34·0	36·8	None
2·5	89·2	155·6	66·4	—	+

As the adjuvant factor only appears to operate after the need of the fungus for vitamin B_1 has been satisfied, it was not allowed for in calculating the results [cf. Sinclair, 1939], as it was assumed that the vitamin supply was acting as a limiting factor under the conditions of the experiment, so that the growth of the fungus was in fact a measure of the vitamin present.

The adjuvant action of potato does not appear to be due to the same causes as that of blood; it is

not due to buffering; cultures with and without potato both reach a *p*H value of 4·0 at the end of incubation. Neither is it due to nitrogenous compounds; in the absence of asparagine, potato and excess vitamin give very little growth as compared with blood and excess vitamin. This is seen in Table 10, which also shows the additive effect of potato and blood when supplied together; this is further evidence that the factors in potato and blood are distinct.

Table 10. *Growth of mycelium on blood and on potato*

	Additions to medium			Growth of mycelium	
Vitamin B_1 (μg.)	Potato (g.)	Blood (ml.)	Asparagine (mg.)	(mg.)	Excess growth in presence of both factors (mg.)
5	—	—	40	77	—
—	0·15	—	40	38	—
—	—	1	40	29	—
5	0·15	—	40	121	6
5	—	1	40	136	30
5	0·15	1	40	188	44
5	0·15	—	—	12	—
5	—	1	—	115	—

It is possible that the adjuvant action of potato observed in these experiments is due to the 'factor Z' described by Robbins [1939]. It is interesting in this connexion that royal jelly from a queen bee cell, which has been reported to be rich in pantothenic acid [Pearson & Burgin, 1941] had no stimulating action whatever upon *P. blakesleeanus*, either in the presence or absence of excess vitamin B_1.

F. *The anomalous action of green sprouts.* White sprouts from potatoes kept in the dark (Table 5) were found to have a high vitamin content. All attempts to repeat this work on green sprouts, however, failed completely. In presence of green sprouts the fungus did not grow at all; there was no visible mycelium, and the *p*H of the medium was unchanged. A second inoculation of fungus spores into the same flask failed like the first. It seems likely, to judge from the high vitamin B_1 content of white sprouts and young leaves, that green sprouts also have a high content, but this cannot be proved, because of their toxic action on the fungus.

It was thought that the toxic action might be due to the solanine in the sprouts; accordingly solanine and solanidine were supplied to the fungus under a variety of conditions, but with very little effect. 2 mg. solanine produced a slight diminution of growth, but smaller quantities no effect at all; the same was true of 4 mg. and smaller quantities of solanidine. There was only one exception: in a single case complete inhibition of growth occurred in a culture containing white sprouts and 2 mg. solanine. It cannot be pretended that the toxic action of green sprouts is explained, but it may be noted that the thiochrome method also failed on green sprouts; the pepsin digest, which was coffee-coloured, gave no thiochrome reaction at all.

DISCUSSION

The vitamin B_1 content of potato tubers is important from the point of view of nutrition, as sources of this vitamin are somewhat restricted in a war-time diet. It appears from this work that mature tubers of the variety Majestic have a vitamin B_1 content, in autumn, of about 140 μg./100 g. On this basis a daily ration of 12 oz. of potatoes, if properly cooked in their skins, would supply 470 μg. of vitamin, or nearly half the usual estimate of the daily requirement of the vitamin (1 mg.). Potatoes, which are eaten by the great majority of people, are at any rate not a negligible source of the vitamin, particularly in the autumn and winter months.

The vitamin B_1 content of the young tubers increases continuously during the growing season; from this fact, and from the very high vitamin content of potato leaves, it can be presumed that the vitamin is synthesized in the leaves in summer and transferred to the young tubers as they grow. In winter the vitamin apparently remains in the tuber till sprouting begins, when it moves out of the centre of the tuber into the sprouts; but further evidence is needed on this point.

It is interesting to note that, in contrast to ascorbic acid, which is usually concentrated in the skin and adjacent layers of fruits and vegetables, vitamin B_1 in potatoes is concentrated in the centre of the tuber rather than in the skin.

The adjuvant action, which is due to some factor or combination of factors not fully explained, appears in tubers only in certain definite physiological states, e.g. in old fully sprouted, and young developing, tubers, between April and August. Adjuvant factors in potato have previously been described [Robbins, 1939], but information is lacking as to the age or physiological state of the tubers from which they were extracted. It can definitely be said that the adjuvant action of potato is due to a different cause from that of blood, but the factor responsible cannot be identified with any previously described.

SUMMARY

1. The *Phycomyces* method of estimation is valid for determining the amount of vitamin B_1 in potatoes.

2. The vitamin B_1 content of Majestic tubers at the time of lifting (September) is about 140 μg./100 g.

3. During the growing season the vitamin B_1 content of tubers increases continuously up to the time of lifting of maincrop potatoes.

4. The vitamin B_1 content of stored tubers falls during the winter and spring; the greater part of the loss takes place in spring, and is apparently due rather to sprouting than to storage, especially as white sprouts have a higher vitamin B_1 content than the tuber which produces them.

5. The vitamin B_1 content of young potato leaves is very high.

6. An adjuvant factor of undetermined nature is present in the centres of tubers between April and August, and always present in the skin layer.

7. Green sprouts completely inhibit the growth of the fungus.

I should like to express my thanks to the following: to F. C. Bawden, under whose auspices the work was carried out, and N. W. Pirie, for advice; to P. H. Gregory for potato samples and help in planning the experiments; to H. M. Sinclair for a culture of *Phycomyces blakesleeanus*; to my brother, A. P. Meiklejohn, for calling my attention to the possibilities of the method, and to E. M. Crook and D. J. Higgons for supplies of solanine and solanidine.

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The Total Nitrogen Content of Egg Albumin and Other Proteins

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The question of amino-acid analysis became one of increasing importance in this laboratory during 1933, when the isolation of proteins from various forage grasses had been successfully accomplished and evidence for their content of the dietetically essential amino-acids was clearly desirable.

Work has continued without interruption since then, but at times progress has been slow on account of the laborious nature of many of the incidental investigations that we have felt called upon to undertake. During the first two years some preliminary studies of the better-known methods for base and dicarboxylic acid analyses were made by Miller [1935; 1936]; these served to emphasize the large amount of humin formation that attended the hydrolysis of our relatively impure leaf products, and little further progress was made until the origin of this humin had been satisfactorily established by Bailey [1937] and Lugg [1939]. The observation that cystine and, to a lesser extent, tyrosine can condense, like tryptophan, with the furfural derived from pentosan-like impurities during acid hydrolysis to form humin led Lugg, in continuation of work started in Adelaide, to investigate the reliability and limitations of the better-known indirect methods for estimating these three amino-acids, and also methionine [cf. Lugg, 1939]. Meanwhile, Tristram [1939] had explored the applicability to these impure leaf proteins of the Block small-scale gravimetric method of base analysis; progress had been made in an attempt to improve the Foreman method of dicarboxylic acid analysis; and the separation of the monoamino-acids by the Dakin-Fischer methods had been explored. With the experience thus gained it was possible to carry out partial amino-acid analyses of a series of protein preparations from forage crops [cf. Chibnall, 1939].

At this stage in our work we were not specifically interested in obtaining strictly quantitative values for the dicarboxylic acids and bases, for such a refinement was considered unnecessary in view of the somewhat impure nature of our leaf products; all that we had set out to attain was an analysis for each product comparable, from a nutritive point of view, with those already in existence for many seed and animal proteins. During 1936–8 however Bergmann & Niemann reported partial analyses of several proteins, and on the results of these they formulated certain stoichiometric laws governing protein structure. Two of the proteins they had selected for consideration, egg albumin and haemoglobin, had already been submitted to dicarboxylic acid analysis in this laboratory, and our results, which we were fairly certain were still several per cent too low, were higher than theirs and definitely not in keeping with the general conclusions they had drawn. Moreover, in agreement we believe with many others, we were not satisfied with the values they had assigned to the molecular weight of the average amino-acid residue.

In 1938 therefore it was decided to attempt to perfect our analytical methods for the dicarboxylic acids and the bases so as to obtain values which we could feel were reliable to 2% or less. In this we have been as successful as we could reasonably expect, and our investigations, which are still in progress, will be reported in later papers. Meanwhile the bearing that these new results have on the structure of the proteins concerned has been discussed in a recent lecture [Chibnall, 1942], and the interpretation was based in part on a value for the molecular weight of the average amino-acid residue computed from data which include, *inter alia*, the total N of the protein. As it is essential that this latter value be accurately determined we were led to the investigations reported in the present communication.

On reviewing the more recent literature dealing with the preparation and analysis of proteins we found that in many instances the recorded value for the total N of the protein concerned was significantly lower than that accepted by earlier reputable analysts such as Osborne and Sörensen, and the disagreement was especially marked in the case of egg albumin. Now it is common knowledge that the two above-mentioned workers used methods of analysis which they had proved by experience were reliable, and the implication that the work of the later investigators might be untrustworthy needed demonstration, for the position left us not only in doubt as to the value to be used in the computation of the average residue weight, but, as will be discussed later, it shook our confidence in the results of many of the more recent amino-acid analyses of protein hydrolysates and physicochemical studies of proteins in solution.

Osborne invariably determined N on the air-dried sample, as he found that, in general, the anhydrous material was too hygroscopic to be handled without special precautions. For the Kjeldahl analysis he used about 0·1 g. protein, 20 ml. H_2SO_4 and a drop of mercury, the heating being continued for 4–5 hr. after clearing and the oxidation completed by adding a few small crystals of $KMnO_4$ immediately after the gas to the burner had been turned off. Sörensen & Höyrup [1915–17] took for analysis an amount of egg albumin solution corresponding to not more than 20 mg. protein-N and digested this with 20 ml. H_2SO_4, 5 g. K_2SO_4 and 50 mg. Cu wire. The digest cleared in 2–4 hr. and the heating was continued for a further 5 hr. The still boiling liquid was then oxidized by adding cautiously 1·5 g. of dry pulverized $KMnO_4$ in 3 equal lots. Both these protein chemists therefore found from experience that prolonged heating of the digest was necessary for an accurate analysis.

In recent years there has been a natural desire to shorten the time period of digestion and this has led to the introduction of new catalysts such as selenium, its dioxide, or metallic selenates, all of which have been shown to be very effective in promoting the rapid oxidation of many forms of organic-N [cf. Bradstreet, 1940]. These modifications, often applied on a micro-scale, have been shown in many instances to give a satisfactory recovery of N if the heating be continued for less than 1 hr. after clearing, and the notion that the time lag inherent in the older methods had been thereby overcome has no doubt been fostered by the undoubted success that has attended most of the rapid micro-methods of organic analysis introduced by Pregl. When these modified Kjeldahl methods are applied, however, to many materials of biological origin (e.g. wheat, flour, seeds, etc.) there is no absolute check on the results obtained, and in the protein and amino-acid field the uncritical acceptance of their trustworthiness is apparent from the data collected in Table 1 and the discussion which follows.

Table 1. *Nitrogen content of certain proteins, as recorded in the literature*

(All values are calculated on an ash-free and moisture-free basis; those in parentheses indicate the ranges with different samples. K indicates Kjeldahl analysis; D, Dumas analysis.)

Protein	Total N (%)	Reference
Egg albumin, heat coagulated	K 15·55 (15·51–15·59) D 15·6 (15·57–15·63)	Osborne & Campbell [1900]
Egg albumin, heat coagulated	K 15·71 (15·61–15·83)	Vickery & Shore [1932]
Egg albumin, native protein	K 15·60 (15·61–15·59)	Taylor, Adair & Adair [1932]
Egg albumin, heat coagulated	K 15·16	Jones & Moeller [1928]
Egg albumin, heat coagulated	K 15·12 (15·03–15·23)	Calvery [1932]
Egg albumin, native protein	K 15·44 (15·1–15·7)*	Hendrix & Dennis [1938]
Egg albumin, native protein	K 15·36 (15·2–15·6)*	Hendrix & Dennis [1938]
Egg albumin, heat denatured	K 15·27	Bernhardt [1940]
Pepsin, twice recrystallized	K 13·95 (13·8–14·1) D 14·8 (14·63–14·83)	Northrop [1939]
Pepsin	K 15·4	Calvery, Block & Shock [1936]
β-Lactoglobulin, recrystallized	K 15·3†	Palmer [1934]
β-Lactoglobulin, 4 times recrystallized	K 15·14, 15·26‡	Pedersen [1936]
β-Lactoglobulin, thrice recrystallized	? 14·35	Winnick [1942]
β-Lactoglobulin, thrice recrystallized	K 15·6	Cannan [private communication]
β-Lactoglobulin	K 15·6	Sörensen & Palmer [1938]

* Ranges of repeat analyses of 1 sample. † Arnold Gunning method. ‡ Gunning-Pregl micro-method.

Osborne & Campbell [1900] made 4 preparations of what they considered to be pure coagulated egg albumin and obtained concordant values for total N by both the Kjeldahl and Dumas methods (Table 1). Sörensen & Höyrup [1915–17] discussed the precautions necessary to obtain a maximum value by the Kjeldahl method for the protein-N present in an $(NH_4)_2SO_4$ solution of the recrystallized protein, and concluded (p. 181) that 'The factor by which the weight of protein nitrogen has to be multiplied to give the weight of the egg albumin dried over solid potassium hydroxide *in vacuo* at indoor temperature is nearly equal to 6·4 and this does not apply solely to soluble crystallizable egg albumin, but also to denatured egg albumin and eventual middle terms between these two substances'. A factor of 6·4 corresponds to 15·63 % N. More recently Vickery & Shore [1932] have obtained a mean value of 15·71 % N for 3 samples of thrice recrystallized protein, while Taylor, Adair & Adair [1932] record a mean value of 15·60 % N for 2 samples of protein that had been crystallized 7 times. In contrast to these high values, which show reasonable agreement, there are others, much lower, which exhibit considerable irregularities. All these latter appear to have been obtained from samples of recrystallized egg albumin, or of heat-coagulated products derived from them; hence one is led to infer that either the analytical procedures employed must have been faulty or the workers concerned have not realized that the anhydrous products are extremely hygroscopic. Calvery [1932] analysed many preparations of the coagulated protein and recorded a mean value of 15·12 % (Table 1). Rather surprisingly this result is referred to later [Calvery, Block & Shock, 1936] in the following terms: 'It has been previously definitely established that the nitrogen content of crystalline egg albumin when dried at 103–110° and corrected for ash is 15·4 %.' Hendrix & Dennis [1938] made a very large number of analyses of 2 samples of crystalline protein, and also of denatured products obtained from them by the action of dilute alkali, dilute acid and by shaking. Sörensen & Höyrup's technique was employed, and the products were dried to constant weight *in vacuo* at 110° before analysis. The results in each case exhibited considerable irregularities and were submitted to statistical treatment. The values for the native proteins are given in Table 1; those for the alkali-denatured (2 samples), acid-denatured and shaking-denatured products, were 15·21, 15·05, 14·86 and 14·95 % N respectively. Robinson & Hogden [1941] determined the percentage N in 66 samples of heat-coagulated proteins from blood serum by a micro-method using $SeOCl_2$ as a catalyst, the heating being continued for only 20 min. after clearing. Their analyses were made on material dried to constant weight, and the difficulty of dealing with this on account of its hygroscopic nature was admitted; the results ranged from 15·4 to 16·2 % with a mean of 15·78 % N. No control analysis of a standard protein of known N content was recorded. The above citations are by no means exhaustive. A search of the literature covering the last 15 years, the period during which the micro-Kjeldahl method has come more and more into general use, reveals many instances in which investigators have recorded the results of amino-acid analyses in terms of percentage weight of protein, the values being admittedly based on a determination of total hydrolysate-N. In these cases it is rare to find any mention of the mode of preparation or of the purity of the protein used; all too often its N content is not recorded and no indication is given of the procedure by means of which the hydrolysate-N was determined, except perhaps a simple statement that the micro-Kjeldahl method had been employed. The results given below illustrate the magnitude of the errors which may be incurred if insufficient attention be given to what is one of the simplest of analytical procedures.

EXPERIMENTAL

Protein samples analysed

Egg albumin. We have to thank Prof. R. K. Cannan for 2 samples of thrice recrystallized material [Kekwick & Cannan, 1936]. The coagulated protein was prepared in the following way. 160 g. of air-dried material, containing 26·5 % Na_2SO_4, were dissolved in 1·5 l. H_2O and poured slowly, with vigorous stirring, into 15 l. H_2O at 90°. The coagulated protein was filtered off on a Büchner funnel, the mass removed from the paper, stirred up with 15 l. H_2O at 90° and again filtered. The washing was repeated, first with 15 l. H_2O at 90° and then successively with 2–3 l. cold H_2O until the last few filtrates were free from sulphate. The product was next washed with ethanol and ether, dried at 70°, ground to a fine powder and passed through a small-meshed sieve. The final product sampled reproducibly for moisture after storage in a bottle at room temperature for 2 years.

Edestin. This was prepared from freshly ground hemp seed in the usual way and twice recrystallized from hot 5 % NaCl solution [Bailey, 1942].

Amandin. This globulin is of interest as it gives on hydrolysis an unusually high percentage of amide-N. Osborne & Campbell [1896] record a mean value of about 19·3 % N by the Dumas method, but Osborne [1910] in a later review recalled that earlier workers such as Ritthausen found only about 18·7 % and gave it as his opinion that 19·3 % was probably a little too high and suggested 19·0 %. We have to thank Dr Vickery for a sample of this protein prepared from almond seeds in 1915 by Mr Leavenworth. It contained, ash- and moisture-free, 18·66 % N. The product, as was to be expected after the lapse of so many years, left a considerable insoluble residue when extracted with salt solution, and as we were anxious to obtain good crystalline material for various purposes purification was effected in the following way on the advice of Dr K. Bailey.

50 g. protein, 6·25 g. NaCl, 125 ml. H_2O and 6·25 ml. ethanol were ground to a paste in a mortar. *N*/14 NaOH was then added with stirring until the mixture was just blue to thymol blue (glass electrode *p*H 7·8–8·2). The volume was next adjusted to 1250 ml. with H_2O, the mixture brought to 25° and shaken gently without frothing for ½ hr., care being taken to ensure that all sticky particles had been dispersed. The mixture was then filtered 3 times through pulp that had been washed with distilled water. The filtrate was clear, and some insoluble dark brown material was left on the pad. *N*/10 HCl was added to the filtrate with rapid stirring until there was a definite sign of turbidity, and the solution then carefully adjusted to *p*H 6·3. At this stage the precipitate was not bulky and consisted of denatured protein, as shown by adding NaCl to a test sample and gently warming. The mixture was filtered on a well-washed pad, and to the clear filtrate was added with stirring 1/10 vol. of 95% ethanol. A white bulky precipitate appeared, which redissolved on warming to 35°. The clear solution was now cooled rapidly with stirring to 0°, left for 1 hr. at that temperature and the separated crystalline material centrifuged off as rapidly as possible in cooled cups. The paste thus obtained was stirred up with about 3 times its volume of 10% acetone, cooled to 0° and again centrifuged. The product was washed twice with 50% ethanol, 3 times with absolute ethanol, and then 3 times with acetone, being filtered off the last time and sucked free from all extraneous solvent. The final product was dried *in vacuo*. The yield was 18 g.

Haemoglobin. We have to thank Prof. E. J. Cohn for a sample of horse carboxyhaemoglobin prepared in November 1938 by Singher and Morro of the Harvard Medical School. It had been thrice recrystallized, heat coagulated and washed free from chloride. As received the product contained a small amount of fibrous material, undoubtedly filter pulp, which was removed by sifting.

β-Lactoglobulin. We have to thank Prof. R. K. Cannan for two 50 g. samples. The twice recrystallized protein had been coagulated by heat, washed free from inorganic salt and dried through ethanol and ether.

Excelsin. We have to thank Dr K. Bailey for a sample of the recrystallized protein [Astbury, Dickinson & Bailey, 1935].

Casein. This was prepared by the method of Cohn & Hendry [1923], except that the solution was clarified by filtration through paper pulp instead of being passed through a Sharples centrifuge.

Insulin. A crystalline sample was obtained from Boots Pure Drug Company Ltd., Nottingham.

Pepsin. We have to thank Dr J. St L. Philpot for 2 small samples of crystalline material.

Methods of analysis

Moisture and ash contents of proteins. The material for analysis was first finely ground in an agate mortar. For the determination of the moisture content a sample was dried in an oven at 102° to constant weight [Brand & Kassell, 1942]. In general this was attained in 3–5 hr. and the further loss on heating overnight did not exceed 0·1%. The ash content was determined by heating a sample of the air-dried material in a muffle at 550–600° to constant weight.

Kjeldahl incineration. Na selenate was first used as a Kjeldahl catalyst in this laboratory in 1935, and our notebooks show that prior to the present investigation, which was started in 1938, we had made use of a digestion mixture consisting of 20 ml. H_2SO_4, 10 g. K_2SO_4 and 25 mg. Na selenate. In addition 0·65 g. $CuSO_4.5H_2O$ was occasionally added. The recorded results for good recrystallized edestin were 18·4±0·1% N, and for coagulated egg albumin 15·3±0·1% N. When we decided in 1938 to make an amino-acid analysis of these two proteins the difference between our values and those quoted by Osborne came into sharp focus and we realized that our analytical methods needed overhaul. The trouble was finally traced to the short period of digestion (2–3 hr. after clearing) we had been employing and the following procedure was found, after a long trial of many, including those of Osborne, Sörensen, Pregl ($HgSO_4$), and Peters & Van Slyke [1932], to give the most satisfactory results with proteins and protein hydrolysates.

Our experience confirms that of Osborne and many others that oven-dried proteins are too hygroscopic for convenient handling, and we have invariably used air-dried material if sufficient were available for a separate determination of the moisture content. The sample for analysis, which should not contain more than 10–15 mg. N, is weighed out in a small glass flat-bottomed cup, stoppered if there be need to dry to constant weight. After weighing, the cup is dropped carefully into a long-necked 250 ml. Kjeldahl flask in such a way that no protein is scattered; about 5 ml. H_2O followed by 20 ml. N-free H_2SO_4 are added and the mixture boiled gently on the stand until charring commences. The flask is then removed from the stand, cooled slightly and 5 g. catalyst mixture added. The latter is prepared by grinding to a fine powder 80 g. K_2SO_4, 20 g. $CuSO_4.5H_2O$ and 0·34 g. Na selenate. The flask is then returned to the stand and the contents gently boiled. The digest clears in 15–30 min. and the heating is continued fairly vigorously for another 8 hr. or, if convenient, overnight. The cooled digest is finally made up to standard volume and an aliquot withdrawn for NH_3 estimation. It is essential, of course, that control blanks for the complete procedure be carried out at frequent intervals. In the case of protein hydrolysates an aliquot containing about 0·1 mg. N is withdrawn and incinerated in the usual micro-Kjeldahl tube, using 2 ml. acid and 0·5 g. catalyst mixture. The procedure then follows that given above, except that the whole digest is used for the NH_3 estimation.

Van Slyke, Hiller & Dillon [1942] have recently drawn attention to the difficulty of determining the N content of lysine by Kjeldahl analysis. According to our experience low values will always be obtained with this amino-acid unless the digest is boiled much more vigorously than is necessary when dealing with a protein rich in lysine such as lactoglobulin. Using a sample of *dl*-lysine monohydrochloride which gave 15·28% amino-N in 30 min. we have obtained a theoretical recovery of N under our usual conditions provided that the digest is boiled briskly on an electric heater for about 12 hr. Using a gas burner the recovery is irregular (95–100%) and it is advisable to add $HgCl_2$ to the catalyst mixture (32 mg./g.). A theoretical recovery of N can then be obtained in 4–5 hr.; in 2 hr. [cf. Weissman & Schoenheimer, 1941] we obtained 98·5%. We confirm the finding of Vickery (private communication) that histidine requires 8 hr. digestion for a theoretical recovery of N. In our experiments with casein and egg albumin (see Table 2) we obtained 15·3% N for each protein when the digest was heated for no longer than ½ hr. after clearing. Similar results were obtained when using the digestion mixture of Campbell & Hanna [1937].

Lysine (especially) and histidine are no doubt responsible for the much longer heating period required to obtain a maximum recovery of N.

Estimation of ammonia. The usual Pregl micro-distillation apparatus was used. Haack micro-burettes, graduated to 0·05 ml. and reading accurately to 0·01 ml., were used for the *N*/70 NaOH and HCl respectively. The latter was made up in 20% ethanol with Tashiro's indicator according to the directions of Conway & Byrne [1933].

DISCUSSION

A summary showing the reproducibility of our results is given in Table 2. It will be seen that our values for egg albumin are in agreement with those of Vickery & Shore, and slightly exceed those of Osborne and of Sörensen: we find also, in confirmation of Sörensen, that there is no significant difference in the values for the native and the heat-coagulated protein. We would tentatively suggest that the lower and irregular values recorded by Hendrix & Dennis [1938] are due in large part to the fact that they worked with anhydrous material. In our attempts to do this we have invariably found that both the native and heat-coagulated proteins are so hygroscopic that 2–3% of moisture are taken up if the sample is exposed for only a few minutes in a desiccator that has been recently opened to the air of the laboratory; in other words, the anhydrous products have desiccating properties and compete with the desiccant for any water vapour present. Anhydrous edestin does not exhibit this peculiarity to such a marked degree and we note that Hendrix & Dennis's value for this protein (18·70% N) is in excellent agreement with Osborne's and our own. Anhydrous insulin [cf. Miller & du Vigneaud, 1937] and anhydrous pepsin, on the contrary, are extremely hygroscopic and we have found it convenient to use the air-dried material. Although only small amounts of crystalline pepsin were available we have had no difficulty in obtaining comparable results by the Kjeldahl and Dumas methods (cf. Northrop's results given in Table 1). When sending the 2 samples of this protein Dr Philpot informed us that they might contain, as is usual, some non-protein-N; we mention this because a somewhat higher value for total N (15·4%) has been recorded by Calvery, Herriott & Northrop [1936]. Crystalline enzymes and protein hormones are usually isolated in small amount after an extensive procedure, so that the investigator naturally begrudges the material that must be allocated to elementary analysis. We think it timely therefore to issue a warning that such products, when anhydrous, are likely to be extremely hygroscopic and need special precautions in handling. During our investigations of egg albumin we sent samples of the heat-coagulated product to a firm of micro-analysts for determination of the moisture content by drying at 100° *in vacuo*. The results, obtained by what we believe to be a standard procedure, ranged from 6 to 7%, whereas we had ourselves obtained 11% when the product was dried at atmospheric pressure for 4 hr. at 102° in a weighing bottle, the stopper of which was replaced before removal from the oven.

In general, it will be seen that our results confirm the higher values recorded by earlier authorities who used macro-methods. Our value for amandin (18·76%) is in keeping with Osborne's surmise that his own, determined by the Dumas method, was a little too-high. In the case of excelsin we had only

Table 2. *Nitrogen content of various proteins*

(Values given in parentheses indicate the ranges of analysis; D indicates Dumas analysis.)

Protein preparation	Air-dried material: Moisture content %	Air-dried material: Ash content %	No. of determinations	N, moisture- and ash-free, %
Egg albumin, native protein A	13·3	26·5	8	15·76 (15·69–15·80)
Egg albumin, heat coagulated A	11·0	0·44	7	15·76 (15·71–15·79)
			6	D 15·72 (15·69–15·84)
Egg albumin, heat coagulated B	9·8	0·5	7	15·74 (15·67–15·81)
Edestin, Sample A	11·2	0·05	7	18·65 (18·62–18·70)
Edestin, Sample B	11·1	0·2	6	18·70 (18·66–18·76)
β-Lactoglobulin	6·7	0·28	6	15·58 (15·53–15·67)
Casein	10·2	0·75	8	15·73 (15·72–15·75)
Excelsin	8·4	0·1	4	17·94 (17·84–18·02)
Amandin, Sample A	9·3	0·1	2	18·77 (18·76, 18·78)
Amandin, Sample B	8·1	0·94	2	18·75 (18·71, 18·78)
Carboxyhaemoglobin (horse), Sample A	12·7	—	2	16·80* (16·82, 16·78)
Insulin	10·0	1·24	2	15·54 (15·53, 15·54)
Pepsin, Sample A	10·87	1·2	1	14·95
			1	D 14·7
Pepsin, Sample B	9·94	1·2	1	14·78
			1	D 14·83

* Ash not deducted.

a small amount available and it was necessary to analyse the anhydrous material. The values obtained show wider variations than we expect when using air-dried material and we see no reason to doubt the slightly higher value (18·17 %) reported by Osborne & Harris [1905] for a sample of good crystalline material. Taken as a whole our results demonstrate that in recent years many investigators, whose interests have naturally been focused on the main aspects of their research, have tended to regard the determination of protein-N as a simple matter demanding no special precautions and have paid insufficient attention to the technique of Kjeldahl analysis. These findings are disturbing, for it is becoming more and more customary in amino-acid analysis, especially when only small amounts of protein are available, for the investigator to employ what he regards as moisture-free material, or alternatively, to take an unknown amount of the air-dried product and compute the weight of moisture-free and ash-free protein from a determination of total N in the hydrolysate, using as a conversion factor Osborne's or some other value for the N content of the protein. The latter procedure is clearly at the mercy of the Kjeldahl analysis, and if the technique employed leads to a low result the computed weight of protein is in error, as must also be the results of all subsequent amino-acid estimations. A similar criticism will apply to physico-chemical studies of proteins in solution, wherein the amount of protein present is computed from a Kjeldahl analysis of an aliquot.

SUMMARY

1. Attention is drawn to the fact that in recent years many protein chemists have been employing a technique for the Kjeldahl estimation of N in proteins and protein hydrolysates which gives low results; this is especially true if micro-methods have been used. Instances are cited in which recorded values for the N content of proteins are significantly lower than those obtained by earlier authorities such as Osborne & Sörensen.

2. It is shown that these low values are due to two causes: (1) The digestion period has been too short: with proteins and protein hydrolysates it is necessary to continue the heating for 8 hr. or more after the digest has cleared. (2) Certain proteins (e.g. egg albumin, pepsin and insulin), when anhydrous, are so hygroscopic that they cannot be handled without special precautions; moisture and N contents therefore should be determined, whenever possible, on separate samples of air-dried material.

3. Using the technique described the following values have been obtained for the N content of the moisture- and ash-free protein: egg albumin (native and coagulated), 15·76 %; edestin, 18·7 %; β-lactoglobulin, 15·58 %; casein, 15·73 %; amandin, 18·75 %; insulin 15·54 %; horse carboxyhaemoglobin (moisture- but not ash-free), 16·8 %.

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Critique of the Foreman Method for the Estimation of the Dicarboxylic Acids in Protein Hydrolysates

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The lime-ethanol method of Foreman [1914*a*] for the estimation of aspartic and glutamic acids in the products of protein hydrolysis has found wide application, and in the hands of later workers has undergone various modifications in detail. Foreman recited the conditions that he considered necessary for the quantitative precipitation of the dicarboxylic acids as their Ca salts from the amino-acid mixture obtained by hydrolysing the protein with HCl. He pointed out that cystine and tyrosine, as well as small amounts of chloride and basic material might also be carried down. The lime-ethanol precipitate was separated by filtration, dissolved in water, Ca^{++} removed as oxalate, Cl^- as AgCl and basic substances with phosphotungstic acid. The excess of the latter and any $SO_4^{=}$ was next removed with baryta, the clarified solution taken to dryness and then extracted with glacial acetic acid. The two dicarboxylic acids remained insoluble and were estimated by differential analysis for C, H and N. The acetic acid extract contained any pyrrolidonecarboxylic acid formed from glutamic acid during the various treatments applied, as well as other products, and the former was estimated by the increase in amino-N following strong HCl hydrolysis. In an addendum to his paper he stated that in the light of subsequent work [Foreman, 1914*b*] he believed that if the temperature for all processes of concentration was kept below 45° the amount of pyrrolidone formation would be reduced to a negligible amount.

Dakin [1918; 1920; 1923] hydrolysed the protein with H_2SO_4, removed the mineral acid with baryta, extracted the monoamino-acids with butanol and then precipitated the dicarboxylic acids under the Foreman conditions as their Ba salts, glutamic acid subsequently being isolated as the hydrochloride, and aspartic acid via the Pb salt. Jones & Moeller [1928] applied the Foreman method direct; in most cases they used the Ba salts and found it necessary to make a preliminary precipitation with phosphotungstic acid to prevent the Ba dicarboxylates being heavily contaminated with basic material. These latter workers, who isolated the glutamic acid as the hydrochloride and aspartic acid as the Cu salt, found that in most cases it was necessary to repeat the original precipitation of the Ba salts to free the mixed products from monoamino-acids such as glycine and tyrosine. Calvery [1931] followed the general procedure of Jones & Moeller and pointed out that losses might be incurred if the monoamino-acids were removed by a preliminary extraction with butanol, as recommended by Dakin. The results of all these workers showed a marked improvement over those obtained by the older ester method of Fischer.

In this laboratory some preliminary dicarboxylic acid analyses of cocksfoot protein were made by Miller [1936]. The Dakin procedure for reasons not then apparent gave low results, while that of Jones & Moeller was by no means satisfying, for much of the N in the Ba precipitate remained unidentified. More systematic investigations of the products obtained from various proteins by HCl hydrolysis were started the following year to ascertain the respective merits of the Ca and Ba salts, the best conditions for the first and repeat precipitations with ethanol, and details of technique for the isolation of glutamic acid as its hydrochloride and of aspartic acid as its Cu salt. These were followed by an analysis of myosin [Sharp, 1939], which demonstrated the superior selectivity of the Ca over the Ba salts when precipitated in the presence of much basic-N* and confirmed Calvery's finding that a preliminary extraction with butanol to remove monoamino-acids may lead to loss of dicarboxylic acids, indicating that the precise conditions under which this extraction can be successfully applied are not readily defined. Using the experience thus gained, reasonably satisfactory dicarboxylic acid analyses were made of a series of protein preparations from forage crops [Chibnall, 1939]. On the completion of this latter work we were free to devote our attention to the more interesting problem [Chibnall, 1942; Chibnall, Rees & Williams, 1943] of perfecting our methods of analysis so as to obtain results for both aspartic acid and glutamic acid which we could be reasonably certain were accurate to about 2%.

* In a private communication to one of us (A. C. C.) Mr Foreman stated that in 1914 he was aware of the superior selectivity of the Ca salts and that his choice was deliberate. The contrary opinion of Town [1941] was based on experiments with gliadin, which contains very little basic-N.

EXPERIMENTAL

We analysed a number of proteins of known purity by the procedure then in current use in this laboratory. The protein (40–50 g.) was hydrolysed with 20 % HCl and then passed twice in succession through the lime-ethanol treatment, using each time an aqueous volume of 400 ml. and 9 vol. of 95 % ethanol. Glutamic acid hydrochloride and Cu aspartate were then separated alternately until no more crystalline material could be obtained from the mother liquor. The lime-ethanol treatment, followed by separation of the crystalline derivatives, was then applied another 2 or 3 times, generally from aqueous volumes of 100, 25 and 20 ml. respectively. Later we made an analysis of various normal and malignant tissue proteins, and in the published account of that work the above-mentioned procedure was described in some detail [Chibnall, Rees, Williams & Boyland, 1940].

Table 1. *Aspartic and glutamic acid content of some proteins*

	Total N ash- and moisture-free %	New analysis: Glutamic acid %	New analysis: Aspartic acid %	Literature: Glutamic acid %	Literature: Aspartic acid %	Authority
Casein (1)	15·72	20·7	6·14	21·6	4·1	Dakin [1918]
Casein (2)	15·72	20·7	6·10	—	—	
Edestin (1)	18·65	18·6	10·55	19·2	10·2	Jones & Moeller [1928]
Edestin (2)	18·65	19·36	10·95	—	—	
Cattle haemoglobin	16·65	5·83	8·03	3·5	6·4	Bergmann & Niemann [1937]
Egg albumin (2)	15·75	14·0	7·02	14·0	6·1	Calvery [1931]

The results given in Table 1 show that the method we were then using for the estimation of aspartic acid was superior to those hitherto employed, but that we had affected no improvement in the estimation of glutamic acid. Our cumulative experience nevertheless convinced us that both these estimations were still far from being quantitative, as will be apparent from the following observations:

1. When the precipitated Ca salts are redissolved in water Ca^{++} cannot be removed quantitatively unless an extended treatment be applied. This is due to the fact that at the stage prior to the addition of lime (which generally contains traces of sulphate) the hydrolysate consists of a mixture of amino-acid hydrochlorides, and on precipitating the Ca salts Cl^-, roughly equivalent to about 20–30 % of the HCl present according to our experience, is carried down presumably in the form of the additive compounds noted by Anslow & King [1927] and by Cannan & Kibrick [1938]. Consequently on subsequent removal of Ca^{++} as oxalate a stage is ultimately reached when the regenerated HCl prevents further precipitation of Ca oxalate and $CaCl_2$ remains in solution. At a later stage, when glutamic acid hydrochloride and Cu aspartate are being removed alternately, the salting out of the former is hindered by the $CaCl_2$ present and the product is often contaminated with a small amount of $CaSO_4$. These difficulties, which can be overcome by treatment, would not arise when working to the original conditions of Foreman, as he removes both Cl^- and SO^{++} at an early stage; nevertheless according to our experience the removal of these ions should be delayed until the maximum amount of Cu aspartate has been separated, otherwise heavy losses of aspartic acid may be incurred on account of the insolubility of Ag and Ba aspartates.

2. The Cu salt of natural *d*(+)-aspartic acid crystallizes from water in clusters of fine blue needles; these can be washed with cold water and dried via ethanol and ether, when they attain a constant air-dry weight in a few hours. The moisture content, determined by heating to 130° for 3 hr., is 29·4 %, corresponding to 4·5 H_2O, and if the atmospheric humidity is low this may fall to about 28·5 % but not lower. Adequate data in support of this statement, which confirms the early work of Ritthausen [1869] and of Hofmeister [1877], will be given in a subsequent communication, and it may be pointed out here that heating the air-dried salt at 140–150° to constant weight, a procedure employed by Abderhalden & Weil [1911] to demonstrate that it contained 5 H_2O, leads to decomposition. We are unable to substantiate the claim of Bergmann & Niemann [1937] that when the air-dried salt is heated for 6 hr. at 105° the moisture content is reduced to 15·62 %, equivalent to $2H_2O$. (Found: moisture 14·4, 14·9 %.) The Cu salt of racemic aspartic acid crystallizes in fine needles of the same habit and colour as the natural salt and has about double the solubility of the latter in water (30 mg./100 ml.) at 20°: air-dried it also contains moisture equivalent to 4·5 H_2O. When pure the blue colour of both the air-dried salts has a characteristic violet tinge that persists even when the material is finely ground. In the many dicarboxylic acid analyses that have been discussed above it was seldom that products of this purity were obtained without resort to recrystallization

and in this respect our experience appeared to be similar to that of Jones & Moeller [1928], who mention that the moisture content of their air-dried products often varied from the equivalent of 4·5 H_2O. Under the microscope our products showed the presence of irregular nodular masses as well as the typical needles of Cu aspartate, and the greater the proportion of the former the less did the colour of the finely ground material match that of pure Cu aspartate. Table 2 gives the analysis of two such products, Cu and N being determined on the anhydrous material.

Table 2. *Characteristics of some Cu-aspartate fractions*

Sample	Colour	H_2O %	Cu %	N %
(1) From edestin	Dull blue	27·8	32·6	7·35
(2) From edestin	Dull blue with distinct greenish tinge	22·0	29·7	7·59
(3) Pure air-dried Cu aspartate	Blue with violet tinge	29·4	32·7	7·19

Sample 1 might well be regarded as pure, yet on recrystallization under appropriate conditions some impurity was removed. In the analyses quoted in Table 1 it has been necessary to recrystallize all the samples of Cu aspartate except the first two main crops from each hydrolysate of casein. The rejected material contained Cu aspartate however and the quoted values for the aspartic acid content of proteins concerned must have been low. To appreciate the steps which led to the recognition of this impurity, and its avoidance in later work, it is necessary to recite briefly the method in use at the time for the separation and purification of the Cu aspartate.

When the solution of mixed amino-acids recovered from the lime-ethanol precipitate, or of the mixed hydrochlorides obtained from them after a preliminary removal of glutamic acid hydrochloride, was treated at the boil with excess $CuCO_3$, a fine scum of varying shades of olive and grey green was generally the first product to separate. If this occurred before the removal by filtration of the excess $CuCO_3$ the Cu aspartate that crystallized out from the filtrate on cooling was fairly pure, as in sample 1 (Table 2). More often, however, the scum did not separate until after filtration, and very occasionally it was possible to remove this by another filtration before crystallization of the Cu aspartate. The scum thus removed was always green and in some cases, especially when small end fractions were being dealt with, it often contained traces of yellowish material. Usually, however, the scum and aspartate separated together and in the case of end fractions very impure products were thereby obtained, as in sample 2 (Table 2). This particular sample was dissolved in hot water with the aid of a few drops of HCl, boiled with excess $CuCO_3$ and filtered hot. The filtrate, as it cooled, deposited a green flocculent precipitate which was removed and washed in the usual way. The yield was 18·3 % and, dried at 100°, the product contained 29·9 % Cu and 7·6 % organic-S. This latter finding gave us at once the clue to our difficulty, for it suggested that during the lime-ethanol treatment cystine was undergoing dismutation [Lavine, 1936] to give the sulphinic or sulphonic acid, perhaps also cysteic acid, all of which dibasic acids give insoluble Ca salts under these conditions. Our experience with casein, which was a very pure sample containing only 0·24 % cystine, tended to confirm this, for the first two large crops of Cu aspartate did not need recrystallization. It will be recalled that Foreman [1914*a*] showed that cystine would be precipitated under the conditions of his lime-ethanol procedure. To obtain further evidence we oxidized some cystine by the method of Friedmann [1903], using insufficient Br to effect a quantitative conversion to cysteic acid. On making the Cu salt according to his directions a scum separated at once from the hot filtrate, and on elutriating this with water we obtained a series of products ranging in colour from dull blue to olive and grey green, typical of those we had encountered in our dicarboxylic acid analyses.

3. Foreman mentioned that tyrosine would be precipitated with the Ca salts of the dicarboxylic acids and we accordingly made it a custom to remove as much of this amino-acid as possible by direct crystallization at the stage immediately prior to the lime-ethanol treatment. Even so the material recovered from the Ca precipitate, after being worked over to remove the major part of the glutamic and aspartic acids, would always yield small amounts of crystalline tyrosine on appropriate treatment [cf. Chibnall *et al.*, 1940]. Moreover, the end fraction, from which no further amount of crystalline amino-acids or derivates could be separated, always gave a strong Millon test for tyrosine and also a strong Sakaguchi test for arginine. We had also noted that when these dicarboxylic acid fractions, after being reduced *in vacuo* to a small volume, were concentrated on the water-bath in the presence of HCl to the incipient crystallization of glutamic acid hydrochloride some substance was decomposed to give small amounts of NH_3. It is possible, from the work of Damodaran & Ramachandran [1941], that the substance concerned was serine, although our experience suggests that the free amino-acid is much more stable to hot strong acid than its phosphoric acid ester. Our final end fraction therefore, which reduced to an intractable syrup, must have contained tyrosine, arginine, serine and perhaps also its decomposition products,

as well as cystine and its dismutation products. It is from these end fractions, not always as well attenuated as those obtained during the analyses referred to in Table 1, that certain earlier workers have isolated what they have believed to be hydroxyglutamic acid. As we show in Appendix 2, we encountered such products in a few of our earlier analyses, but these always yielded relatively large amounts of aspartic acid and sometimes of glutamic acid on appropriate treatment.

Now as experience was gained during the course of this research the relative amount of N that remained in the dicarboxylic acid end fraction became progressively less, with an accompanying increase in the amount of aspartic acid isolated. The improvement was due to the more effective removal of the various amino-acids mentioned above, whose presence can retard the crystallization of Cu aspartate. This salt is very insoluble in cold water, yet 0·33 g. will dissolve readily when stirred into 100 ml. of water at 20° containing 1·9 g. of Cu serine. Such solubility effects illustrate the underlying fallacy of the so-called Brazier [1930] method for the separation of the products of protein hydrolysis. This method, in fact, was first suggested by Town [1928; 1936; 1941] who has recently drawn attention to its limitations.

All the above-mentioned observations convinced us that our final end fraction, though representing no more than about 1 % of the total protein-N, still contained aspartic acid and perhaps also glutamic acid, and the first problem ahead of us was the complete removal of cystine at an early stage, when the hydrolysate was still acidic, so as to prevent its dismutation during the lime-ethanol treatment.

Removal of cystine as cuprous mercaptide prior to the lime-ethanol precipitation

During the course of our research on the glutamic acid content of normal and malignant tissue proteins we had occasion to repeat some of the experiments of Kögl & Erxleben [1939]. These workers hydrolysed the tissue material with HCl and after removing the excess of acid in the usual way they clarified their hydrolysate by treatment with Cu_2O, a reagent first introduced by Hlasiwetz & Habermann [1873], who found it very effective for the purpose and also a cheap substitute for Ag_2O in the removal of the residual chloride. Certain of our hydrolysates clarified in this way were afterwards passed through the lime-ethanol procedure, and we observed that the various crops of Cu aspartate subsequently isolated were free from all traces of the scum mentioned earlier, were of high purity and did not need recrystallization. This suggested to us the possibility, which we found subsequently had already been demonstrated by Rossouw & Wilken-Jorden [1935], that the Cu_2O, or more correctly the Cu_2Cl_2, was reducing the cystine to cysteine, which was then being precipitated as the cuprous mercaptide [Vickery & White, 1932]. At this time we already had in view an outline of the extended procedure that we hoped to apply to a dicarboxylic acid and base analysis of proteins, and Rossouw & Wilken-Jorden's treatment for the removal of cystine was unsuitable for our purpose in that it would introduce reagents whose subsequent removal would present difficulties. The direct use of Cu_2O was accordingly investigated in some detail, the work being completed and the results applied to edestin, egg albumin and zein before the publication of a paper on somewhat similar lines by Lucas & Beveridge [1940].

A short account of the experiments leading to the procedure finally adopted is given in Appendix 1. To effect the complete precipitation of the cystine as the cuprous mercaptide of cysteine it is necessary to add an excess of Cu_2O, thereby bringing about an almost complete removal of HCl as Cu_2Cl_2 and reducing the acidity of the hydrolysis mixture to about *p*H 3·5. As a result a small amount of the more insoluble monoamino-acids, viz. methionine, tyrosine and probably also leucine and phenylalanine, separate as such, or as copper complexes, and contaminate the mercaptide precipitate. Careful search, however, has shown that there is no loss of dicarboxylic acids or arginine. The new treatment has been applied to analyses of edestin, egg albumin and β-lactoglobulin, full details of which are given in a later paper [Chibnall *et al.*, 1943]. As was to be expected, there was a great improvement in the purity of the Cu aspartate subsequently isolated, and only very occasionally has it been necessary to recrystallize the product before satisfactory analytical data could be obtained. In these analyses a more complete removal of the interfering monoamino-acids such as tyrosine and serine carried down in the initial precipitate of the Ca dicarboxylates was effected by applying the third and subsequent lime-ethanol treatments from a larger aqueous volume than had been our custom in the past.

The results for edestin and egg albumin are given in Table 3 under the heading '1st complete lime-ethanol treatment'. The new procedure has given in both cases a satisfactory increase in aspartic acid, but no increase at all for glutamic acid, suggesting that the separation of this amino-acid as hydrochloride was well nigh complete under the old conditions. That both the dicarboxylic acids have been effectively removed is shown by the fact that in the egg albumin analysis the ultimate mother liquor contained only 0·95 % of the total protein-N and of this some 40 % was non-amino-N, almost certainly basic-N since precautions had been taken to prevent the conversion of glutamic acid to pyrrolidone-

carboxylic acid. Moreover, the Millon test for tyrosine and the Sakaguchi test for arginine were strongly positive. These results convinced us that we had brought our treatment for the isolation of glutamic and aspartic acids from the lime-ethanol precipitate to workable limits; even so we could not regard our new values as quantitative until we had ascertained whether any significant amount of either of the respective Ca salts had escaped precipitation and had thus passed into the lime-ethanol filtrate.

Examination of the lime-ethanol filtrate for dicarboxylic acids

As will be more fully discussed in Appendix 3, the Ca salts of both aspartic acid and glutamic acid, under the conditions of our lime-ethanol procedure, have residual solubilities that are subject to the concentration and the degree of racemization of the acid concerned and to the concentration of Cl^-. At an early stage in the research, before the interdependence of these three factors had been established, we had determined solubility factors by submitting an aqueous solution of each natural acid to the necessary lime-ethanol treatment and these suggested that our values for aspartic acid and glutamic acid should be raised by about 0·3 and 0·7 % of the weight of protein respectively. Such solubility effects were larger than we had anticipated and we were not willing to concede them without a more direct proof based on the isolation of the two amino-acids from the lime-ethanol filtrates.

Accordingly we undertook an extended analysis of gliadin. After hydrolysis with 20 % HCl the hydrolysate was reduced in volume and saturated with gaseous HCl; this caused the separation of a large crop of glutamic acid hydrochloride which was subsequently recrystallized from water [Osborne & Guest, 1911]. The collected mother liquors were then passed through the lime-ethanol procedure in use at the time; as this did not include the preliminary application of Cu_2O the various small crops of Cu aspartate were heavily contaminated with the scum mentioned in an earlier section, and needed careful recrystallization. When the lime-ethanol precipitate had been worked over to completion we had isolated in all 45·82 % of glutamic acid and 1·34 % of aspartic acid (*vide* Table 3), values that from the standpoint of analytical technique are comparable with those of edestin (2) quoted in Table 1. Now at the stage when the first precipitation of the Ca salts was made the hydrolysate contained amino-acids, other than dicarboxylic acids, representing 48 % of the total protein-N. All of these other amino- (and imino-) acids, as well as any residual dicarboxylic acids due to solubility effects, passed into the collected lime-ethanol filtrates, and our object was to devise methods whereby these other amino-acids could be progressively removed so as to give a final mother liquor in which the residual dicarboxylic acids would be present in sufficient concentration to permit their isolation by a repeat application of the lime-ethanol procedure. The various filtrates and the final dicarboxylic acid end fraction were collected, reduced *in vacuo* to remove ethanol and then freed from inorganic ions (Ca^{++}, Cl^- and traces of $SO_4^=$). The clarified solution was next concentrated to incipient crystallization, whereby several successive crops of mixed monoamino-acids (leucine-tyrosine-phenylalanine) were removed. The mother liquor was then extracted 12 times successively with an equal volume of warm butanol [Miller, 1936] to remove further amounts of monoamino-acids, a treatment that extracts but little proline. This was followed by a precipitation under the usual conditions with phosphotungstic acid, which carried down the bases and much proline. The filtrate was freed from phosphotungstic acid by extraction with amyl alcohol-ether and from excess HCl by repeated evaporation *in vacuo*. At this stage the solution contained only 12·8 % of the total protein-N in the form of amino-acids other than dicarboxylic acids, and on repetition of the lime-ethanol procedure we were able to isolate a further 1·1 % of glutamic acid and 0·06 % of aspartic acid. This experiment demonstrated quite clearly the relatively large solubility of Ca glutamate under the conditions of our modified Foreman lime-ethanol procedure, especially when some of the acid is present in racemized form, as in this gliadin hydrolysate [Chibnall *et al.*, 1940, Table 1]. The solubility of Cu aspartate on the contrary appeared to be small, a result accepted at the time with reserve on account of the omission of the Cu_2O procedure and of possible losses arising from the conditions under which the base precipitation with phosphotungstic acid had been performed.

Having made this preliminary survey we were in a position to devise a more suitable, albeit a more laborious, procedure which has been applied in turn, with minor modifications suggested by experience, to hydrolysates of zein, edestin, egg albumin and β-lactoglobulin. Full details of the last three of these analyses are given in a later paper [Chibnall *et al.*, 1943] and all that need be quoted here are the summarized results for edestin and egg albumin given in Table 3, which permit a direct comparison with the earlier ones given in Table 1. In the new analyses it was possible to reduce the amount of non-dicarboxylic acid-N from about 70 % to about 14 % of the total protein-N before repetition of the final lime-ethanol procedure and, as in the case of gliadin, substantial amounts of (partially racemized) glutamic acid were subsequently isolated as hydro-

chloride. The new procedure has also led to the isolation, as Cu aspartate, of significant amounts of aspartic acid. The analyses of zein and β-lactoglobulin gave similar results and, as will be discussed in later papers, there is reason to believe that our present procedure gives values for the aspartic acid and glutamic acid contents of the proteins concerned that are accurate to within 2%. These findings show without any equivocation that the lime-ethanol method of Foreman, as applied by us and by all other workers in the past, must have given results that were far from quantitative and hence of no value whatsoever from the point of view of the Bergmann-Niemann hypothesis.

Table 3. *Redetermined aspartic and glutamic acid contents of some proteins*

Protein	Method of isolation	Non-dicarboxylic acid-N present when Ca salts were first precipitated (% total protein-N)	Glutamic acid (% of protein)	Aspartic acid (% of protein)
Gliadin	Direct as hydrochloride	—	33·53	—
	1st complete lime-ethanol treatment	48·0	12·29	1·34
	2nd complete lime-ethanol treatment	12·8	1·10	0·06
		Total	46·92	1·40
Edestin (3)	1st complete lime-ethanol treatment	68·8	19·34	11·24
	2nd complete lime-ethanol treatment	13·9	1·19	0·71
		Total	20·53	11·95
Egg albumin (2)	1st complete lime-ethanol treatment	70·39	13·89	7·74
	2nd complete lime-ethanol treatment	21·3	2·11	0·36
	3rd complete lime-ethanol treatment	12·0	0·0	0·03
		Total	16·00	8·13

Appendix 1. Procedure for Removal of Cystine as the Cuprous Mercaptide of Cysteine. (By K. BAILEY)

For these experiments wool and edestin were chosen because they contained very high and average contents of cystine respectively. The wool was taken from a raw Cotswold fleece, cleaned and prepared in a manner previously described [Bailey, 1937]: it contained 0·13% ash and, ash- and moisture-free, 16·55% N. Both this and the edestin were hydrolysed for 24 hr. with 20% HCl, and the excess HCl removed by evaporation under reduced pressure in the usual way. The hydrolysate was then made up to standard volume and aliquots withdrawn for determination of N by micro-Kjeldahl and of S by alkali fusion [Bailey, 1937]. Cu_2O was prepared by reduction of Fehling's solution with glucose, washed free from all inorganic ions and dried via ethanol and ether.

For the mercaptide precipitation solutions containing 0·7–0·9 g. N/100 ml. in the case of wool, and 0·8–1·1 g. N/100 ml. in the case of edestin, were employed. Portions of the hydrolysate were treated with Cu_2O under varying conditions, which are described below in apposition to individual experiments; after cooling to 10° the precipitate was centrifuged and washed 6 times with warm water. The washings were collected, reduced *in vacuo*, cooled to 10°; the small amount of material that separated was centrifuged off and added to the main precipitate. For N and S analyses the latter was either dissolved in hot HCl or dried in acetone and ground to a fine powder, depending on the amount available.

Wool. The following experiments were performed:

(1*a*) The hydrolysate was shaken with excess Cu_2O at 15° for 30 min., cooled to 10° and the mercaptide precipitate separated without delay.

(1*b*) As in (1*a*), but the reaction was allowed to proceed overnight before the separation.

(1*c*) As in (1*a*), but the reaction temperature was raised to 45° and the excess Cu_2O added in small portions over a period of 30 min.

(2) The Cu_2O was added slowly to the hydrolysate at 45°, small samples of which were removed from time to time and clarified by centrifuging to test whether the addition of a further amount would produce any visible separation of mercaptide. In this way the considerable excess of Cu_2O added in 1*a*–1*c* was avoided and the supernatant liquid was green instead of deep blue.

(3) This was a large-scale repetition of (1*c*) under conditions considered most suitable for the purpose, and was designed as the starting-point of an extended amino-acid analysis. The hydrolysate, containing 18·4 g. N in 2 l., was maintained at 45–50° whilst a suspension of Cu_2O in 500 ml. of water was poured in slowly with rapid stirring until a test sample showed that excess Cu_2O was present and the supernatant liquid was deep blue. Stirring was continued for another hour and the mixture was then kept at 10° overnight.

The results are given in Table 4. It will be seen that for the complete removal of cystine the reaction temperature must be raised to 45°, and an excess of Cu_2O must be present (Exp. 1*c*, 3). Under such conditions the acidity of the solution is reduced to about *p*H 3·0–3·5 by removal of HCl, and the supernatant liquid becomes deep blue. The N carried down is in excess of that due to cystine, a point discussed later.

Edestin. Based on the experience gained with wool hydrolysates the following experiments were performed:

(4) The conditions were as in (3).

(5) The starting-point of a large-scale comprehensive dicarboxylic acid and base analysis described in detail in a later communication [Chibnall *et al.*, 1943]. The conditions were as in (3).

(6) The hydrolysate was heated with a small amount of Zn and HCl for 1 hr. and then neutralized with NaOH at *p*H 6. The humin thus precipitated was separated and the filtrate adjusted to *p*H 4 with HCl. Excess Cu_2O was then added at 45° and the mixture agitated for 1 hr.

(7) The procedure of Graff, Maculla & Graff [1937] was employed. The hydrolysate was first reduced with Zn and HCl, adjusted to *p*H 5 with Na acetate, humin removed, the solution adjusted to *p*H 4 with acetic acid and then treated with Cu_2O. The mercaptide precipitate was washed with a citrate-acetate buffer at *p*H 6.

The results given in Table 4 show that in Exp. 4 and 5 the mercaptide-S is greatly in excess of the non-methionine-S, assumed to be cystine-S, which amounts to 43·5% of the total S. Under the conditions of (6) and especially of (7) the mercaptide-S approaches the expected value for cystine-S, though in the latter case the mercaptide-N is higher than the equivalence claimed by Graff *et al.* [1937].

The ideal procedure for our large-scale amino-acid analysis would have been one in which the precipitate contained only the mercaptide of cysteine, Cu_2Cl_2 and excess Cu_2O, together with any humin not already removed at an earlier stage. That of Graff *et al.* comes nearest to fulfilling these conditions, but was unsuitable for our purpose in that it would introduce into the hydrolysate reagents whose subsequent removal would present difficulties. The technique used in Exp. 3, 4 and 5 was satisfactory in so far as removal of cystine was concerned, yet at the time these were performed we were puzzled by the large excess of S and N carried down. In this connexion our findings were in full keeping with those published recently by Lucas & Beveridge [1940], who used a somewhat different technique and found that their mercaptide precipitate was heavily contaminated with extraneous N. As these workers determined cystine-S and not total organic-S they were apparently unaware that non-cystine-S was carried down.

The excess organic-S in our mercaptide precipitates was clearly due to methionine, yet the reason for this was not immediately apparent. When a solution of this amino-acid was treated with Cu_2O under comparable conditions only 4% of the original N was carried down. Moreover, Bailey [1937] had already shown that the methionine content of a protein first hydrolysed with HCl is the same as that of the native protein when the determination is made by the volatile iodide method; secondary reactions, such as homocystine formation during hydrolysis, were thus excluded. It was not until the mercaptide precipitates obtained in our large-scale analyses of egg albumin and edestin had been submitted to detailed investigation that we were able to explain why they contained more organic-S and N than had been expected. The reason is that the addition of excess Cu_2O to the hydrolysate, which is necessary for the complete precipitation of the cystine as the cuprous mercaptide of cysteine, removes the major part of the HCl present as insoluble Cu_2Cl_2 and the acidity of the solution is thereby reduced to about *p*H 3. Such conditions are favourable for the direct crystallization of certain of the more insoluble monoamino-acids, especially methionine, tyrosine, leucine and perhaps phenylalanine [Hill & Robson, 1934; Baptist & Robson, 1940], and it is these monoamino-acids, or possibly their copper complexes, together with humin, that are responsible for the excess N and organic-S present in the mercaptide precipitates. Appropriate treatment has shown that arginine [Lucas & Beveridge, 1940] and the dicarboxylic acids are not concerned, a matter of importance in our work.

Table 4. *Composition of various mercaptide precipitates*

Exp. no.	Total S minus free SO_4-S g.	Mercaptide-S as % total protein-S	Filtrate from mercaptide: S minus SO_4-S as % total protein-S	Total N in hydrolysate g.	Mercaptide-N as % total protein-N	Cystine-N as % total protein-N
(*a*) Wool						
1*a*	0·0716	89·8 (diff.)	10·2	—	—	—
1*b*	0·0716	89·6	13·7	0·3748	10·03	8·32
1*c*	0·0716	100·6	0·6	0·3748	11·74	8·32
2	0·640	90·6	9·4 (diff.)	3·28	11·6	8·32
3	3·607	97·3	3·6	18·41	12·4	8·32
(*b*) Edestin						
4	0·039	74·1	29·0	0·7903	2·62	0·85
5	0·541	67·3	32·7 (diff.)	11·554	2·20	0·85
6	0·027	51·9	48·1 (diff.)	0·5745	1·83	0·85
7	0·027	45·5	54·5 (diff.)	0·5745	1·34	0·85

Appendix 2. On the Possible Presence of Hydroxyglutamic Acid in Protein Hydrolysates

As Nicolet & Shinn [1942] state in a recent paper, the history of β-hydroxyglutamic acid is a peculiar one. In their introduction, these workers give a review of the position that had developed since Dakin [1918] announced the isolation of this substance from casein, and it is unnecessary to recapitulate the various evidences in the present communication, except to point out that many workers who have attempted to prepare β-hydroxyglutamic

acid for experimental purposes have been unsuccessful or have obtained products which they believed to contain considerable amounts of the desired substance, but have not adequately characterized it. Applying their new periodic acid oxidation method for the estimation of threonine and serine in protein hydrolysates, together with that of Van Slyke, Hiller, MacFadyen, Hastings & Klemperer [1940] for 'total hydroxyamino-acids', Nicolet & Shinn [1942] find no evidence for the presence of hydroxyglutamic acid in the three milk proteins, casein, β-lactoglobulin and lactalbumin. In view, however, of the claim of Gulland & Morris [1934] that this hydroxyamino-acid is indeed present in casein, albeit only to the extent of 0·3% as opposed to Dakin's original 10·5%, Nicolet & Shinn were not prepared to deny its existence in this protein, though they maintained that the amount present cannot be greater than that claimed by Gulland & Morris. Meanwhile Dakin [1941] has stated that further research on the acid that he isolated from casein, particularly his failure to convert it to ketoglutaric acid, or glutaconic acid derivatives, and its limited reaction with periodic acid, make it seem certain that his product cannot be a β-hydroxy-acid. This admission of Dakin may be regarded as closing the issue concerning β-hydroxyglutamic acid, yet the position as it now stands is not entirely satisfactory, for Dakin is exploring the possibility that his product is some other new amino-dicarboxylic acid. This raises at once a matter of great importance to those who are interested in the number of free carboxyl-groups in the intact protein molecule, for these can be computed on the one hand from electrometric titration of the protein itself and on the other hand from dicarboxylic acid and amide-N analysis after hydrolysis [cf. Chibnall, 1942]. Consequently if Dakin's product should indeed be a new dicarboxylic acid instead of, as we believe, a mixture of known amino-acids and products derived from them, it must be taken into account in drawing up an estimate of total dicarboxylic acids given on hydrolysis.

When we embarked on our extended investigations of the Foreman method we naturally made careful search for hydroxyglutamic acid by the glacial acetic acid and Ag salt procedure of Dakin. In our early experiments we frequently obtained, like other workers, syrups that proved intractable. Yet occasionally, and in general by procedures different from that of Dakin, we separated crystalline or granular products whose composition suggested the possible presence of this hydroxy-amino-acid; on further treatment, however, these always yielded significant amounts of aspartic acid and often of glutamic acid. Later, when we had evidence that the Foreman lime-ethanol treatment was bringing about the dismutation of cystine to give dibasic acids, we found that all of our 'hydroxyglutamic acid' fractions contained significant amounts of organic-S and hence of cystine dismutation products, which were clearly in part responsible for the fact that the fractions often gave values for C, H and N which approximated closely to those of hydroxyglutamic acid itself. Since the introduction of the Cu_2O treatment and the development of our present procedure for the separation of aspartic and glutamic acids we have not encountered any fractions of this type whatsoever and are convinced that all the proteins that we have analysed, viz. edestin, egg albumin, β-lactoglobulin, zein, gliadin and casein, do not contain any other dicarboxylic acid. We believe that the products isolated by Dakin, and by Gulland & Morris, were mixtures of aspartic acid (especially), glutamic acid, dibasic dismutation products of cystine, serine and serine decomposition products, the proportion in which these occur varying from sample to sample. To enable us to elaborate the thesis further it will be convenient to give first of all in some detail the separation and subsequent analysis of certain 'hydroxyglutamic acid' fractions obtained in three of our earlier dicarboxylic acid analyses.

Egg albumin. This analysis was made in 1938, and the final results have already been quoted in Table 1. 51·193 g. of air-dried coagulated material, equivalent to 49·8 g. of moisture- and ash-free protein, were hydrolysed with 400 ml. of 20% HCl for 24 hr. and the excess HCl removed by repeated evaporations *in vacuo*. The acid humin was then removed, the solution and washing reduced to 500 ml., lime added to *p*H 6·8–7·0 and the alkali humin removed. The clarified solution and washing were then reduced to 200 ml. and left overnight at 0°: 1·098 g. crude tyrosine separated and were removed. The lime-ethanol treatment of Foreman was then applied twice in succession from an aqueous volume of 500 ml., the precipitated Ca salts filtered and washed, redissolved in water and Ca^{++} removed. Glutamic and aspartic acids, equivalent to 11·85 and 5·91% of the weight of protein (w/p), were separated as the hydrochloride and Cu salt respectively, and the clarified mother liquor passed through the lime-ethanol treatment from an aqueous volume of 110 ml. The precipitated material was freed from Ca^{++} as before and the clarified solution reduced to 50 ml. This was allowed to stand under slightly reduced pressure in a desiccator, when 3 crops of crystalline material (0·38, 1·09 and 0·404 g. respectively) separated and were removed. The collected material contained tyrosine (≡0·14% w/p by colorimetric estimation) and on treatment gave glutamic acid (≡1·04% w/p) and aspartic acid (≡0·86% w/p) as hydrochloride and Cu salt respectively.

The mother liquor, after removal of the above-mentioned 3 crops, was reduced to a small volume and treated with ethanol. Amorphous material (fraction 1) separated, which was washed with ethanol and ether and then dried to constant weight *in vacuo* (1·716 g.). It was non-hydroscopic, was freely soluble in cold glacial acetic acid, gave the colour test for tyrosine and contained 2·4% ash ($CaSO_4$); other analytical data are given in Table 5 and are reminiscent of those given by the 2 samples of impure S-containing nitrogenous acids isolated from casein by Gulland & Morris, one of which contained C, 31·3; H, 5·5; N, 10·6; S, 6·7% respectively. Fraction 1 contained 9·02% N, all in the amino-form, but after heating for 1 hr. at 120° the latter value had fallen to 5·72%, reminiscent of Dakin's hydroxyglutamic acid. Titration to *p*H 7·3 (blue tint with bromothymol blue) showed that only 68·4% of the N could be referred to dicarboxylic acids [*vide* Chibnall *et al.*, 1940]. 1·674 g. of fraction 1 yielded by the usual treatment 0·2571 g. of aspartic acid and 0·7114 g. of glutamic acid as Cu salt and hydrochloride respectively. The clarified mother liquor was passed through the lime-ethanol procedure, using an aqueous volume of 10 ml., the precipitate removed, freed from Ca^{++} and the solution reduced to 3 ml. On standing 0·244 g. of crystalline material (fraction 2) separated and was removed. The product was insoluble in glacial acetic acid and gave a positive colour test for tyrosine. The lime-ethanol filtrate was freed from ethanol and Ca^{++}, joined with the mother liquor from fraction 2 and taken to dryness *in vacuo*. The product (fraction 3, wt. = 0·4733 g.) was slightly hygroscopic, dissolved freely in glacial acetic acid, contained 4·5% ash ($CaSO_4$) and gave a strong colour test for tyrosine. Fraction 1 therefore,

which was freely soluble in glacial acetic acid, had yielded aspartic acid (≡0·345% w/p), glutamic acid (≡0·86% w/p), fraction 2 which was insoluble in glacial acetic acid, and fraction 3 which was freely soluble in glacial acetic acid. Fractions 2 and 3 were not investigated in further detail, but their analyses, quoted ash-free in Table 5, and especially their mode of preparation, show that they must be mixtures of several products.

Protein material from normal ox heart. The analysis of this material (47·2 g.) has already been described in detail [Chibnall *et al.*, 1940]. The amino-acid solution recovered from the Ca salts precipitated in the 5th lime-ethanol treatment was reduced to a small volume *in vacuo*; on standing 0·1232 g. of crude tyrosine separated. The mother liquor was then concentrated slowly to a syrup in a vacuum desiccator and after 2 days a few drops of ethanol were stirred in. On standing 0·78 g. of crystalline material separated. For convenience this is referred to in the present communication as fraction 4. It contained 1·8% ash ($CaSO_4$), was insoluble in glacial acetic acid, gave the colour test for tyrosine, and on drying *in vacuo* at 40° gave the ash-free analysis quoted in Table 5. The values for C, H, N and mol. wt. (by titration) come within the limits quoted by Dakin [1918] for his various samples of hydroxyglutamic acid. 0·4 g. of fraction 4 gave on treatment 0·27 g. of crystalline Cu aspartate, and the aspartic acid recovered from this was found to be almost completely racemized, the rotation in 10% HCl being $[\alpha]_D = +0·7°$ as against +25·6° given by the natural *l*(−)-amino acid. No glutamic acid hydrochloride could be separated from the mother liquor, which was not treated exhaustively, however, as our interest at the time was centred on the rotation of the isolated aspartic acid.

Protein material from metastases in liver of a woman with carcinoma of the colon. A fairly detailed analysis has already been described [Chibnall *et al.*, 1940]. The amino-acid solution recovered from the Ca salts precipitated in the 4th Foreman treatment was reduced to a small volume, when 0·396 g. crude tyrosine separated. The mother liquor on further reduction gave 0·4738 g. of crystalline material (fraction 5). This was insoluble in glacial acetic acid, gave a strong colour test for tyrosine, and on treatment gave 0·346 g. of Cu aspartate, the acid recovered from which was racemized; $[\alpha]_D = +4·5°$ in 10% HCl.

conditions for the isolation of his acid and his analytical figures approximate more closely to our fraction 4. We make no claim to have investigated the nature of our products at all exhaustively, for at the time they were isolated we had no such purpose in view, but we believe that sufficient evidence has been adduced to show that all of them must be complex mixtures containing varying amounts of tyrosine, dismutation products of cystine, aspartic acid, and in one case glutamic acid. We have ample evidence moreover that serine is precipitated as its Ca salt under the Foreman conditions, confirming the recent findings of Damodaran & Ramachandran [1941], who also showed that on hydrolysis of substances such as the phosphopeptone from casein (and hence of casein itself wherein the serine is present as phosphoserine) much deamination occurs with the formation of secondary acidic N-free decomposition products. As has been pointed out in an earlier section, the presence of serine will prevent the quantitative separation of aspartic acid as its Ca salt, and we know that the presence of the latter acid can retard the salting out of glutamic acid as its hydrochloride; hence we can legitimately infer that fractions 1 and 3, perhaps also one or more of the other three, contain larger amounts of these two dicarboxylic acids than we actually isolated by the simple procedure employed. In our dicarboxylic acid and base analysis of egg albumin, edestin and β-lactoglobulin [Chibnall *et al.*, 1943] the amount of unidentified N in the respective end fractions from the 2nd series of Foreman operations was very small (*ca.* 0·3% protein-N) and an exhaustive search has convinced us that these do not contain any aspartic acid, glutamic acid or unknown dicarboxylic acid. All these observations convince us that Dakin's products from casein, and also Calvery's [1931] product from egg albumin, were mixtures similar to those described above. Dakin's varying yields can almost certainly be correlated with the fact that he used glacial acetic acid to separate his new acid from residual amounts of glutamic and aspartic acids. Fraction 1 shows clearly that the solvent action of this reagent depends on the physical condition of the material being extracted, and if this be a fine amorphous powder much glutamic and aspartic acids can pass into solution.

Similar criticisms to those given above apply to the substance isolated from casein (1·5 kg.) in very small yield (20 mg.) by Gulland & Morris [1934]. These workers failed

Table 5. *Compositions of 'β-hydroxyglutamic acid' fractions*

Source	Fraction	C %	H %	N %	Organic-S %	Mol. wt. (by titration)
Egg albumin	(1)	31·46	6·10	9·02	4·53	—
	(2)	37·90	6·60	8·88	1·94	164
	(3)	33·33	5·58	6·70	5·20	163
Ox heart	(4)	36·03	5·91	8·84	1·28	168
Human liver	(5)	33·10	5·96	9·70	0·92	162
Hydroxyglutamic acid requires		36·79	5·56	8·59	—	163
Glutamic acid requires		40·80	6·17	9·50	—	147
Aspartic acid requires		36·08	5·31	10·53	—	133
Cysteic acid requires		21·30	4·14	8·28	18·95	169

The analyses given in Table 5 show that under varying conditions products having lower values for C, H and N than those of glutamic acid can be separated, and that if the assumption be made that these are dibasic acids their molecular weights computed by titration range from 162 to 168. Dakin, it will be recalled, used more standard

to separate any product by Dakin's procedure and investigated the N carried down in the $BaSO_4$ obtained on removal with baryta of the H_2SO_4 used in the hydrolysis. Their treatment was extensive and when much extraneous material had been separated they obtained a fraction, referred to above, which was rich in organic-S, showing that

they were dealing with dismutation products of cystine. At a later stage they claim to have isolated 20 mg. hydroxyglutamic acid after a preliminary phosphorylation with phosphoric acid and phosphoric oxide. It may be mentioned that no evidence was adduced to show that any such condensation took place, and the reagent employed may have done no more than aid the attenuation of what was evidently a complex mixture.

Appendix 3. Solubility of the Ca Salts under the Conditions of the Modified Foreman Procedure

Foreman [1914*a*] showed that when an aqueous solution of either aspartic or glutamic acid (1 g. in 20 ml.) was treated with lime and then with 9 vol. ethanol the Ca salt was quantitatively precipitated. Under the conditions of our original modified lime-ethanol procedure, however, all the amino-acids in the protein hydrolysate are present as hydrochlorides, and our early work showed that the $CaCl_2$ thereby introduced into the system raised the small residual solubility of the Ca dicarboxylates. Solubility factors were accordingly obtained from experiments in which the respective dicarboxylic acid hydrochlorides were passed through the procedure then in use, and these were employed as a guide in our researches until 1939, when our attention became focused more sharply on the problem by the claim of Kögl, Erxleben & Akkermann [1939] that the Ca salt of synthetic *dl*-glutamic acid (a mixture of the *l*(+)- and *d*(−)-antipodes) had 10 times the solubility of that of the natural *l*(+)-antipode, and that our procedure was accordingly unsuitable for the isolation of racemic glutamic acid from protein hydrolysates. We were able to refute these claims by appropriate experiments showing that the solubilities of the 2 salts were similar and by the demonstration that our procedure had already led to the isolation of relatively large amounts of partially racemized acid from hydrolysates of gliadin and edestin [Chibnall *et al.*, 1940; see also Graff, Rittenberg & Foster, 1940].

More detailed experiments with the Ca salts of glutamic and aspartic acids were then undertaken to ascertain the effect of the degree of racemization and the concentration of the salts concerned on solubility, and also of the concentration of $CaCl_2$ present. The technique employed was as follows. A weighed amount of the acid was dissolved in about 30 ml. of water and to this was added the necessary aliquot of a standard solution of $CaCl_2$, followed by an excess of CaO. The mixture was stirred for a few minutes and then filtered with suction through a Whatman no. 50 filter paper. The residue was washed repeatedly with small amounts of warm water, the collected filtrates made up to 100 ml. and 9 vol. of 95% ethanol added. The precipitate was filtered off after 10 min. (leaving for longer periods up to 24 hr. made no significant difference), washed with 95% ethanol, and the collected filtrates were taken to dryness *in vacuo*. The residue was then submitted to Kjeldahl analysis, using 20 ml. H_2SO_4; duplicate determinations tallied to 0·2 mg. N. Figs. 1–3 represent graphically the solubilities for various acid and $CaCl_2$ mixtures. Although somewhat meagre in some cases the data taken as a whole permit us to draw certain conclusions and can be put to limited use with protein hydrolysates.

The curves given in Fig. 1 show that the solubility of the Ca salt of *l*(+)-glutamic acid increases with the concentration of $CaCl_2$ and with the initial amount of glutamate. This latter phenomenon can possibly be explained in terms of complex ion formation between the Ca glutamate and $CaCl_2$, for there is evidence for the presence of a double salt of these 2 compounds in the Ca precipitate. The curves given in Fig. 2 suggest that in the presence of $CaCl_2$ the solubility of the *d*(−)-salt is slightly less than that of the natural antipode, and the same is true of the *dl*-salt made

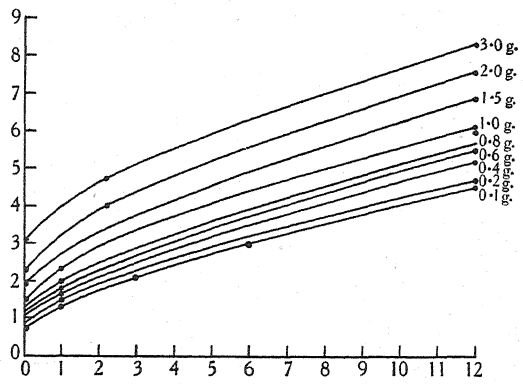

Fig. 1. Ordinates represent the residual solubility of the Ca salt of *l*(+)-glutamic acid when aqueous solutions containing various concentrations of the salt, and of $CaCl_2$, are treated with 9 vol. of 95% ethanol (mg. N per l. of aqueous-ethanol). The abscissae represent the concentration of $CaCl_2$ in the aqueous solution before addition of ethanol (g. per 100 ml.).

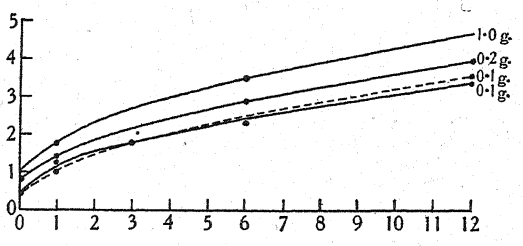

Fig. 2. Similar systems for the Ca salts of *d*(−)-glutamic acid (dotted-line curves) and of *dl*-glutamic acid (full-line curves).

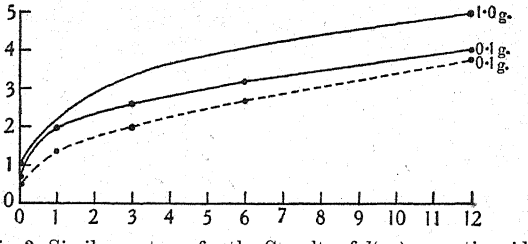

Fig. 3. Similar systems for the Ca salts of *d*(+)-aspartic acid (full-line curves) and of *dl*-aspartic acid (dotted-line curves).

from the synthetic acid, which Kögl *et al.* [1939] have shown to be a mixture of the 2 antipodes. The curves given in Fig. 3 show that similar conclusions apply to the Ca salts of aspartic acid.

From these findings it is possible to compute tentative solubility corrections for a dicarboxylic acid analysis covering no more than the first complete lime-ethanol treatment, provided that the concentration of HCl and of the aspartic or glutamic acid at each precipitation is known. Fortunately the necessary data are available for most of our recent analyses; and those for the glutamic acid of egg albumin are set out in Table 6, the assumption being made that the *l*(+)- and *dl*-salts have independent solubilities.

Table 6. *Solubility corrections for the glutamic acid in egg albumin hydrolysates*

1st complete lime-ethanol treatment								
		Glutamic acid present			Solubility correction		Wt. of glutamic acid (corrected)	
Precipitation no.	Aqueous volume ml.	*l*(+)-acid g.	*dl*-acid g.	$CaCl_2$ present (g./100 ml.)	*l*(+)-acid (mg. N/100 ml.)	*dl*-acid (mg. N/100 ml.)	*l*(+)-acid g.	*dl*-acid g.
1	500	9·0	1·0	1·45	3·75	1·6	0·232	0·101
2	500	8·6	0·8	1·4	3·5	1·5	0·223	0·093
3	500	0·7	0·4	0·4	1·3	1·2	0·081	0·075
4	500	0·25	0·4	0·15	1·0	1·0	0·063	0·063
							0·599	0·332
Isolated during 2nd complete lime-ethanol treatment							0·721	0·60

The solubility corrections suggest amounts of the *l*(+)- and *dl*-acids that are less than those actually isolated during the 2nd complete lime-ethanol treatment [*vide* Chibnall *et al.*, 1943] indicating either that the solubilities had been enhanced by the presence of the other amino-acids in the hydrolysate or possibly that the isolation of the hydrochlorides from the end fraction of the first lime-ethanol treatment had not been quantitative. It is to be noted, however, that the crops of partially racemized glutamic acid isolated from this hydrolysate contain the true racemate (as opposed to the *dl* mixture) of Town [1941], since these give rise to a 3:5-dinitrobenzoyl derivative, m.p. 204° (private communication from Dr Town). The amounts isolated during the second treatment confirm our deduction that the solubility of the salt of the racemic acid is of the same order as that of the *l*(+)-antipode and refute the claim of Kögl *et al.* [1939] to which attention has already been drawn.

In terms of the weight of protein the results for edestin, egg albumin and gliadin are given in Table 7; as in no case have we examined the optical rotation of the aspartic acid the computed values are based on the solubility corrections for the Ca salts of the natural *d*(+)-acid. Considering the assumption made the agreement is reasonable in all cases except that of the gliadin aspartic acid, and the low isolation value here may be due to the unsatisfactory procedure used to cover the various stages leading to the 2nd lime-ethanol treatment.

It is possible to calculate in the same way solubility corrections for the 2nd complete lime-ethanol treatment: these are very small and possible errors of no significance. In terms of the weight of protein the computed values for the above-mentioned proteins, in the order named, are 0·2, 0·13, 0·08% glutamic acid and 0·05, 0·03 and 0·03% aspartic acid respectively.

As in the case of the 3 particular proteins mentioned in Table 7 the computed values are in general slightly less than those found experimentally we have thought it safe to apply similar corrections to such of our earlier dicarboxylic acid analyses, covering only the first complete lime-ethanol treatment, as we consider justified in light of experience.

Table 7. *Comparison of quantities of amino-acids isolated by 2nd lime-ethanol treatments with computed solubility corrections*

	Glutamic acid		Aspartic acid	
Protein	Computed from solubility corrections %	Isolated during 2nd complete lime-ethanol treatment %	Computed from solubility corrections %	Isolated during 2nd complete lime-ethanol treatment %
Edestin (3)	1·26	1·19	0·53	0·71
Egg albumin (2)	1·51	2·11	0·52	0·71
Gliadin	1·19	1·10	0·36	0·06

In the 2 casein analyses the optical rotation of the various crops of glutamic acid hydrochloride was determined, and solubility corrections for the amount of *dl*-acid present have been applied. We believe that the new values

Table 8. *Corrected aspartic and glutamic acid contents of some proteins*

	Glutamic acid			Aspartic acid		
Protein	From 1st complete lime-ethanol treatment %	Solubility correction %	Total %	From 1st complete lime-ethanol treatment %	Solubility correction %	Total %
Casein (1)	20·69	1·31	22·00	6·14	0·54	6·68
Casein (2)	20·67	1·33	22·00	6·10	0·58	6·68
Horse carboxyhaemoglobin	6·05	0·74	6·75	8·76	0·56	9·32
Cattle haemoglobin	5·84	0·52	6·36	8·03	0·56	8·56

for this protein are reasonably quantitative. In the 2 haemoglobin analyses the Cu_2O treatment was not applied and the glutamic acid was not examined, consequently we expect that all the new values will ultimately be shown to be too low.

Finally we should like to mention one point that has puzzled us for some years. Our own extensive experience with the lime-ethanol method of dicarboxylic acid analysis has shown that the first Ca precipitate carries down with it much extraneous N; yet Damodaran [1931], in his modification for determining the dicarboxylic acid-N of proteins on a micro-scale, found that the amount of N carried down was in reasonable agreement with that suggested by the older analyses for the dicarboxylic acid contents of the protein concerned. Our solubility data represented graphically in Fig. 1–3 suggest an explanation of the anomaly. Damodaran hydrolysed the protein with HCl and removed the excess of mineral acid by evaporation to a syrup *in vacuo*, as in a Van Slyke analysis. We find that under these conditions much HCl in excess of that bound as hydrochloride is retained, and to remove this excess it is necessary to add water and again evaporate, repeating the operation 4 or 5 times. At the precipitation stage, therefore, Damodaran's hydrolysate must have contained an excess of $CaCl_2$, which increased the residual solubility of the Ca dicarboxylates and reduced the amount of N precipitated to a value that showed fortuitous agreement with the dicarboxylic acid-N calculated from earlier analyses.

SUMMARY

1. The lime-ethanol method of Foreman for the determination of aspartic and glutamic acids in protein hydrolysates, and the various modifications of it introduced by other workers, have been investigated in considerable detail.

2. During treatment with the lime the cystine present in the hydrolysate undergoes partial dismutation to the sulphinic or sulphonic acids; these are precipitated with the Ca dicarboxylates by the ethanol, together with small amounts of cystine itself, tyrosine, serine and bases.

3. The Ca salts of these cystine dismutation products are very insoluble and interfere with the estimation of aspartic acid as Cu aspartate. To prevent their formation it has been found advantageous to remove all cystine as the cuprous mercaptide of cysteine before the hydrolysate is made alkaline with lime.

4. The solubility of the Ca dicarboxylates under the conditions of the modified Foreman procedure we have used has been investigated by clarifying the lime-ethanol filtrate, removing the bases and a major part of the monoamino-acids, and repeating the lime-ethanol treatment on the final mother liquor. Significant amounts of both aspartic and glutamic acids have been isolated.

5. Based on these findings a new extended procedure has been elaborated which there is reason to believe has given values for the aspartic acid and glutamic acid contents of gliadin, edestin and egg albumin that are accurate to about 2%.

6. The possibility of applying solubility corrections to the dicarboxylic acid results obtained in an analysis covering only one complete lime-ethanol treatment has been investigated. Results for casein, horse haemoglobin and cattle haemoglobin obtained in this way are higher than those hitherto recorded, and it is probable that those for the 2 latter proteins are still too low.

7. Reasons are given for the belief that the 'hydroxyglutamic acid' fractions obtained by earlier workers are complex mixtures containing *inter alia* aspartic acid and dismutation products of cystine.

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The Dicarboxylic and Basic Amino-acids of Edestin, Egg Albumin and β-Lactoglobulin

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The various investigations made in this laboratory during the few years preceding 1938 on the amino-acid composition of leaf proteins have been briefly summarized elsewhere [Chibnall, Rees & Williams, 1943]. These served to convince us that many of the methods of analysis we were then using, in particular those for the dicarboxylic acids and the bases, could not be applied to better characterized proteins with the precision demanded by the Bergmann-Niemann generalizations on protein structure [Chibnall, 1942] until their respective advantages and limitations had been more clearly defined and incidental working losses drastically reduced.

In accordance with this view we have thoroughly explored the Foreman method for the separation of the dicarboxylic acids, and an account has already been given [Bailey, Chibnall, Rees & Williams, 1943] of the various experiences which led us ultimately to devise a satisfactory procedure. This is incorporated in the general scheme of analysis for the dicarboxylic and basic amino-acids which is described in the present paper. Our aim has been to isolate these two groups of amino-acids as pure derivatives and in quantitative yield by step-wise removal without overall loss, and to overcome the solubility factors inherent in all precipitation methods it was necessary to remove also, at the appropriate stages, a large part of the monoamino-acids and proline. In formulating a procedure for doing this we have been guided to a large extent by our experience of the butanol method of Dakin [1918; 1921; 1923] and of the Cu salt method of Town [1928; 1936; 1941]. To anyone who has had experience of the earlier Fischer methods of esterification and distillation the elegance of the Dakin procedure needs no emphasis, for the monoamino-acids are obtained in the convenient form of a white crystalline powder free from both proline and hydroxyproline. In this laboratory Dakin's method has been applied to myosin [Sharp, 1939] and to cocksfoot protein [Lugg; *vide* Chibnall, 1939, p. 162], the esters subsequently being distilled under low pressure through a modern fractionating column. A certain degree of success was obtained, but the method was not proceeded with for two reasons. In the first place we found that small amounts of the dicarboxylic acids and bases were extracted by the butanol. Secondly, we found that extensive diketopiperazine formation occurred, even when the butanol extraction was carried out under reduced pressure, leading to losses amounting to several per cent of the total protein-N. The meagre data quoted by Dakin [1923] in his analysis of zein suggest that much of the N that he was unable to account for was located in the butanol-proline fraction. Our experience of the Cu salt separation has been the same as that of Town himself, in that we have found that the solubility of one particular salt or group of salts can be profoundly influenced by the presence of others. The method must therefore be used with discrimination, and unless circumstances warrant, no exaggerated idea of the purity of the products isolated must be entertained. The procedure for the estimation of the dicarboxylic acids and the bases given below embodies our cumulative experience of these two methods and it leads, incidentally, to the separation of the monoamino-acids in excellent yield but in several fractions, each of which is a mixture. These could be used, either singly or collectively, for the estimation of the indi-

vidual components by the ester method, as had been our original intention, but the new partition chromatographic method of Gordon, Martin & Synge [1941; 1943], which is applied to the hydrolysate without any preliminary fractionation, has proved so strikingly successful that it is doubtful if our products will be required for this purpose.

EXPERIMENTAL

1. *Scheme of analysis*

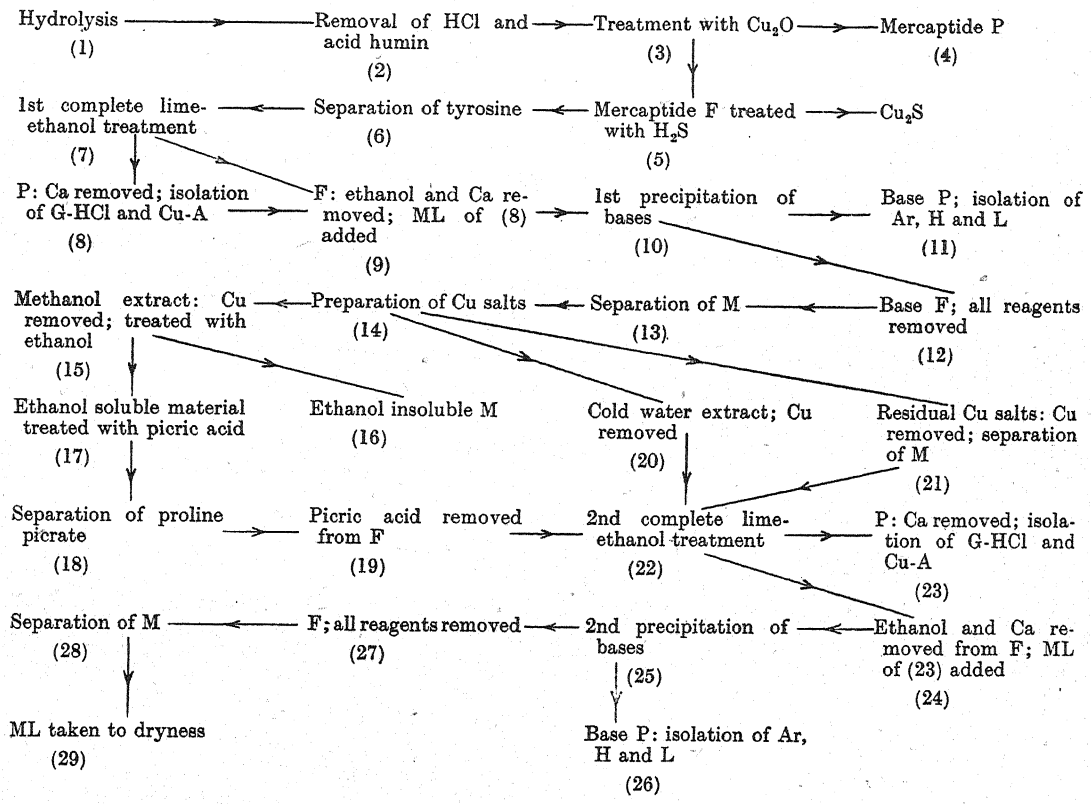
The cardinal point in the procedure is that no reagent may be introduced into the main hydrolysate unless it can be quantitatively removed at a later stage without appreciable loss of N, for in an extended scheme such as we have adopted the working losses are cumulative and can soon amount to several per cent of the total protein-N. It follows that certain reagents either cannot be used at all or only at a stage when there is reason to believe they comply with this provision.

Now it has been the experience of all protein analysts that if H_2SO_4 be used to effect hydrolysis and the mineral acid is subsequently removed with baryta, the resulting $BaSO_4$ obstinately retains N much in excess of that due to humin. We find nevertheless that after the removal of the cystine and the major part of the aspartic acid, i.e. at stage 9, Ba^{++} or $SO_4^{=}$ can be introduced into the hydrolysate and removed in the usual way as $BaSO_4$ without appreciable loss of N, an observation which points strongly to the fact that the excess N carried down when this latter operation is conducted at an earlier stage represents these two amino-acids, very probably in the form of double salts with $BaSO_4$. Dakin [1921] mentioned the difficulty of completely removing Ba^{++} with H_2SO_4 from solutions of Ba

Diagram 1.

Scheme of amino-acid analysis.

Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate; Ar, arginine; H, histidine; L, lysine; M, mono-amino-acids; P, precipitate; F, filtrate; ML, mother liquor.



hydroxyaspartate, but we are unaware of any corresponding citation for the two above-mentioned amino-acids. To avoid these large losses certain workers have preferred to hydrolyse the protein with HCl, remove the excess acid in the usual way

by evaporation *in vacuo* and then free the hydrolysate from Cl^- with Ag_2O. The precipitate of AgCl is much less bulky than the corresponding one of $BaSO_4$, and less excess N is retained, but in our experience it is still appreciable and may amount in some cases to as much as 4% of the total protein-N. Such losses are due to the insolubility of the Ag mercaptide of cysteine, even at fairly high concentrations of H^+ [Bailey, private communication], and of both Ag aspartate and glutamate, for at stage 9 Ag^+ or Cl^- can be removed as AgCl at *p*H 3 without loss of N. According to our experience CuS, Cu_2S and Ca oxalate do not retain N (except humin-N, if present); Cu^+, Cu^{++} and Ca^{++} therefore can be removed from the hydrolysate at any stage as these salts respectively. Lastly, if phosphotungstic acid be used to precipitate the bases, this acid can be removed from both precipitate and filtrate in the usual way with amyl alcohol-ether mixture without loss of N, provided that the aqueous phase be first saturated with ether and ether-water be used for all aqueous washings. If this precaution is not adopted the numerous aqueous washings soon reduce the ether content of the organic solvent mixture to such a low level that a small amount of N is extracted by the amyl alcohol on the Dakin principle.

All of the above-mentioned observations have been taken into account in devising the scheme of analysis set out in Diagram 1. The rationale is as follows. The protein is hydrolysed with HCl, the excess acid and the acid humin removed in the usual way and the clarified solution treated with Cu_2O (3) to precipitate the residual humin and the cystine as the cuprous mercaptide of cysteine. Following the separation of some crude tyrosine (6) the hydrolysate is passed through the first complete lime-ethanol treatment for the isolation of the dicarboxylic acids (7–8). The mother liquor from this is joined with the Ca-free filtrate (9) and the first precipitation of the bases is made with phosphotungstic acid (10); cystine and the major part of the aspartic acid having been previously removed the precipitate (11), according to our experience, consists almost exclusively of basic material. All reagents and the residual Cl^- are next removed from the base filtrate, which is then reduced to a small volume. On standing, a partial separation of the less soluble monoamino-acids (leucine, phenylalanine, tyrosine and methionine) occurs (13). The soluble products are then passed through the Cu salt treatment of Town, when additional amounts of the above-mentioned mixed amino-acids (21), a valine-*iso*leucine fraction (16) and a large part of the proline (18) are separated. These last few stages serve to reduce the monoamino- and imino-acid content of the hydrolysate to between one-third and one-fourth of that present at stage 7, and it is now ready for the 2nd complete lime-ethanol treatment (22). Following this a 2nd precipitation of the bases is made with phosphotungstic acid (25), and as the major part of the proline has been previously removed the precipitate (26) contains very little non-basic-N. All reagents are then removed from the base filtrate and the clarified solution reduced to a small volume, when further crops of monoamino-acids separate (28). The ultimate filtrate is taken to dryness *in vacuo* and granulated with acetone to give a fine white non-hygroscopic powder (29).

It will be seen that this extended procedure has enabled us to make a direct investigation, via stages 22 and 25, of the solubility factors operative during the first treatment for the dicarboxylic acids at stage 7, and the first treatment for the bases at stage 10 respectively. This is, of course, one of the essential features of the analysis, and as we determine the amide-N at stage 2 and the composition of the mercaptide precipitate at stage 4 we have knowledge of the gross weight and total N of the hydrolysis products present in all the various fractions. We are thus able not only to compute the overall losses and indicate from flow sheets at which stages these occur, but we can determine directly the gross weight of products given on hydrolysis of 100 g. of protein, a factor that can also be computed indirectly in terms of the total N and of that in non-peptide form [see Chibnall, 1942].

Needless to say all reagents employed must conform to certain standards of purity. $CuCO_3$ must be free from sulphate, chloride, and other metals; the commercial samples we have tested do not satisfy these requirements and we have prepared our own from A.R. $CuSO_4$. H_2SO_4 must be N-free. 12-Phosphotungstic acid must be not only N-free but also Na-free; few commercial products satisfy this latter requirement. The samples that we have used were not purified through ether by Winterstein's [1898] method as we were not specifically concerned with phosphotungstate solubilities [Van Slyke, Hiller & Dillon, 1942]. Ag_2O and Cu_2O must be washed free from salts and alkali, $Ca(OH)_2$ must be free from sulphate and iron; we have used a commercial sample but the preparation from Ca is recommended.

2. *Analysis of edestin*

An estimation of total N was made at every convenient stage in the analysis [Chibnall *et al.*, 1943] and flow sheets giving details of aliquots withdrawn were kept throughout so that factors were available to correct all values to the weight of protein (W/P) or to the total protein-N (T.P.N.) as the case may be. The numbers at the head of each section given below refer to the stage numbers in Diagram 1.

Details of analysis

1, 2. 61·741 g. of moisture- and ash-free protein, containing 11·52 g. of N or 18·65% were hydrolysed for 23 hr. with 400 ml. 20% HCl. The excess HCl was removed by evaporation *in vacuo* to a gum; water was then added and the mixture again reduced to a gum, the latter operation being repeated 6 times in all. Experience has shown that under these conditions all excess HCl is removed, and the amount present is no more than that required to bind the amino-acids and ammonia. Ammonia-N, obtained by distillation of an aliquot with alkali at *p*H 12·5 in a micro-Kjeldahl apparatus, was 10·42% T.P.N. This value is, of course, much in excess of the true amide-N, which is 9·49% [Gordon *et al.*, 1941; Chibnall, 1942]. The acid humin was too small to be separated, and it was removed later with the alkali humin in the mercaptide precipitate at stage (3). Data for the composition of the humin (Table 1) were obtained from a sample made by very cautiously treating an aliquot of the solution with lime to *p*H 7·5. Maximum precipitation was not obtained at *p*H 7 with NaOH. The separated humin was washed and dried; N and ash ($CaSO_4$) were determined.

3, 4. The main solution was treated with Cu_2O as described in a previous paper [Bailey *et al.*, 1943]. The Cu^+-free filtrate was colourless, showing that all humin had been removed. The precipitate contained S≡67·3% total protein-S and N≡2·20% T.P.N. The S is in excess of that due to cystine and must represent methionine. Colorimetric estimation before and after the precipitation showed that the precipitate contained by difference tyrosine N≡0·41% T.P.N. These two latter amino-acids and probably also very small amounts of phenylalanine and leucine must have separated, as such or as copper complexes, from the hydrolysate during the period when the mercaptide mixture was allowed to stand at 10° overnight, for the removal of Cl^- as Cu_2Cl_2 had reduced the acidity to about *p*H 3·5, in which region the solubility of these amino-acids is much depressed [Hill & Robson, 1934; Baptist & Robson, 1940].

Table 1. *Composition of the organic material in the mercaptide precipitate (edestin)*

	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
Humin	0·729	1·16	0·0759	0·61
Cystine (0·2255 g. S)	0·846	1·37	0·0985	0·85
Methionine (0·1385 g. S)	0·644	1·04	0·0605	0·53
Tyrosine	0·247	0·4	0·0191	0·21
Total	2·466	3·97	0·254	2·20

5, 6. The mercaptide filtrate and washings were collected, a few ml. HCl added and Cu^+ removed with H_2S. As the washing of inorganic precipitates such as Cu_2S must be thorough if working losses are to be kept down to the minimum, the details of our method are given fairly fully. The many precipitates of CuS obtained in later stages were treated in a similar way.

The solution is transferred to a Büchner flask fitted with a rubber bung carrying a glass tube, for the entry of H_2S, which reaches nearly to the bottom of the flask, the side limb being closed with a short length of pressure tubing and a screw clip. H_2S is then passed in under a pressure of about 18 in. water and the contents of the flask shaken until the Cu_2S coagulates to leave a clear liquid and no more gas is taken up. The mixture is then filtered on a Büchner funnel, using a hardened paper, and before allowing the precipitate to pack hard it is washed 2 or 3 times with cold water (stirring). Meanwhile the main filtrate is being concentrated *in vacuo* to a syrup to remove H_2S and excess HCl if this be present. The precipitate is removed from the filter paper, transferred to a 250 ml. centrifuge bottle fitted with a rubber bung, shaken vigorously with cold water until it is completely disintegrated and again filtered. This operation is generally repeated twice. The precipitate is next transferred to a 1 l. Pyrex beaker, 300–400 ml. of water are added and the beaker immersed in a boiling water-bath for 30–45 min. The precipitate is then filtered off and the operation repeated 5 or 6 times. Occasionally the washings become colloidal and it is necessary to add a few drops of mineral acid to induce flocculation. The collected washings are then reduced *in vacuo* to a syrup and joined with the main filtrate. The resulting solution is then tested for traces of Cu^+ by passing in H_2S, filtered if necessary from any sulphide or elementary S present, and made up to standard volume for determination of N.

The flow sheets showed that at the end of stage 5 there was no detectable loss of N. The remainder of the solution was treated with lime to *p*H 3·5 and reduced to 200 ml. On standing 4 days at 0° a small crop of crude tyrosine separated; colorimetric estimation showed that 86% of the product was tyrosine [Meyer, Field & Klemm, 1940].

7. The filtrate and washings from the tyrosine, of volume about 1200 ml., were treated with freshly prepared cream of lime until an excess remained undissolved. The mixture was then reduced *in vacuo* to a syrup to remove ammonia. Water was then added to make the volume 400 ml., followed by 3600 ml. of 95% ethanol, which were added quickly with vigorous stirring. The separated Ca salts and excess lime were filtered off without delay on a Büchner funnel, using a hardened filter paper, and the extraneous mother liquor removed by pressing the mass to a hard pad, which was then washed with a little absolute ethanol. Suction was continued until most of the ethanol passed through and the material could be reduced to a friable powder by rubbing with a spatula. Unless this precaution be adopted difficulty will be experienced, on account of the presence of ethanol, in redissolving the Ca salts in a small volume of warm water; the dry salts nevertheless are hygroscopic and the suction must not be continued too long, especially if the laboratory air is somewhat humid. The material was next transferred to a beaker, warm water added with stirring to dissolve the Ca salts, and without removing the residual excess lime the volume was adjusted to 400 ml.; 3600 ml. of 95% ethanol were then added.

The Ca salts were filtered off, washed as before and extracted with about 800 ml. warm water, the small residue of lime being washed 8 times by stirring with 150 ml. water kept near the boil for a few minutes and centrifuging. The clear liquors were collected, Ca^{++} removed as described below with oxalic acid and the mixture centrifuged. The clarified solution was reduced *in vacuo* to 200 ml. and reserved. The Ca oxalate was washed once with cold water, and then 8 times with 150 ml. hot water; the collected washings were cooled to room temperature and kept for some time to permit the recrystallization of the small amount of redissolved oxalate. The latter was removed by centrifuging,

the clear washings collected and reduced *in vacuo* to 50 ml. The small amount of oxalate that separated was centrifuged off and the clarified liquor added to the main solution mentioned above. The volume was then made up to 1 l. and 5 ml. withdrawn for analysis. The total N present (corrected) was equivalent to 20·1 % T.P.N.

Without delay each of the two lime-ethanol filtrates was reduced *in vacuo* to a syrup to remove ethanol and traces of NH_3. The syrup from the first filtrate was then taken up in about 1 l. of water and oxalic acid added until no further precipitation occurred. The Ca oxalate was removed by centrifuging, washed once with hot water and reserved for treatment later. The clear centrifugate was taken to a syrup *in vacuo*, water added and again removed *in vacuo*, this treatment being applied 4 times in all to remove HCl set free from any $CaCl_2$ that had originally been present in solution. The removal of HCl at this stage is necessary, as Ca cannot be completely precipitated as oxalate in its presence, The syrup was again taken up in water and just sufficient oxalic acid added to throw out a small precipitate of Ca oxalate, which was removed by centrifuging and washed once. Both oxalate and washing were reserved. The syrup from the 2nd lime-ethanol filtrate was treated with oxalic acid, the Ca oxalate washed once as before and reserved. The clear centrifugate was reduced *in vacuo* 4 times as before to remove free HCl. Just sufficient oxalic acid was then added to throw out a fairly heavy precipitate of Ca oxalate, which was washed once and reserved. Free HCl was again removed as before, followed by precipitation of the Ca as oxalate. The number of times this operation must be repeated varies with the solution concerned; in the present case 4 treatments were necessary. All the precipitates of oxalate from both filtrates were then collected and washed thoroughly 8 times in succession with 250 ml. boiling water. The combined filtrates, including those from the initial 1st washings, were then reduced *in vacuo*, added to the two main centrifugates and the solution reduced *in vacuo* to 200 ml. Final adjustments for the removal of Ca and oxalate ions were then made, the small precipitate washed and the filtrate and washings made up to 1 l. for the determination of total N.

8. (Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate.) The solution was reduced *in vacuo* to 200 ml., the very small amount of Ca oxalate that separated was filtered off, the filtrate and washing again reduced *in vacuo* to 50 ml., filtered from a further small amount of separated Ca oxalate, and the filtrate and washings transferred to an open dish with about 30 ml. conc. HCl. Evaporation was then allowed to proceed to incipient crystallization on a water-bath. After standing at room temperature overnight the separated material was filtered off on a sintered glass crucible, washed with a little conc. HCl followed by absolute ethanol, and dried off *in vacuo* over NaOH to give G-HCl crop 1.

The filtrate was reduced *in vacuo* 6 times to remove free HCl and taken up in 600 ml. boiling water. Purified $CuCO_3$ was added in excess and the hot solution filtered immediately on a hot funnel to remove the excess carbonate. The filtrate was cooled and kept at 0° for 4 hr. Cu-A crop 1 was filtered off and washed many times with water, followed by ethanol and ether. Cu^{++} was removed from the filtrate with H_2S in the presence of HCl, and the CuS washed. The clarified filtrate and washings were collected, reduced *in vacuo* to 20 ml., transferred to an evaporating basin, a few ml. conc. HCl added and the mixture reduced to 5–6 ml. on a water-bath. On standing for 20 hr. at 0° G-HCl crop 2 separated; this was filtered, washed and dried as described above.

On further treatment in the same way Cu-A crop 2 and G-HCl crop 3 were obtained. Excess HCl was removed from the mother liquor and a 3rd lime-ethanol precipitation made from an aqueous volume of 30 ml. The filtrate was freed from Ca^{++} as described above. The precipitate was redissolved in water and Ca^{++} removed with oxalic acid until the *p*H was reduced to 3·2. The clarified solution was next reduced to about 20 ml. and kept at 0° overnight when a small crop of crude tyrosine, ≡0·30 % w/P, separated. Ca^{++} was then removed from the mother liquor. The clarified solution gave Cu-A crop 3 but no glutamic acid. The lime-ethanol treatment was then applied twice in succession, from aqueous volumes of 10 and 30 ml. respectively. The clarified precipitate, on standing many weeks, gave G-HCl crop 4 containing 4 % ash ($CaSO_4$), but no Cu aspartate could be separated. The final mother liquor contained 0·1173 g. N (corrected), ≡1 % T.P.N. Flow sheets showed that at the end of stage 8 the overall working losses amounted to 25·8 mg. N (actual) or 30·4 mg. N (corrected), equivalent to only 0·27 % T.P.N.

9, 10. The five lime-ethanol filtrates (the last two still contained Ca^{++}), together with the mother liquor mentioned above, were collected, freed from ethanol, and made up to 1 l. The total N, uncorrected, was 7·82 g. For the precipitation with 12-phosphotungstic acid (P.T.A.) two trial runs with a mixture of 50 ml. solution, 50 ml. water and 14 ml. conc. HCl showed that 20 g. P.T.A. in 20 ml. water were in slight excess. 895 ml. of the main solution were accordingly diluted to 1795 ml. with water and 240 ml. conc. HCl added. The whole was warmed to 60° and a warm solution of precipitant (216 g. P.T.A. in 400 ml. water) poured in with stirring. The two trial runs were then added and the mixture allowed to stand for 2 days at room temperature. The precipitate was filtered on a Büchner funnel using a hardened paper, suction being maintained until the material had been pressed down to a hard pad and no further liquid passed. The material, still on the paper, was next puddled to a fine cream with 100 ml. of wash solution (2·5 g. P.T.A. in 100 ml. of *N* HCl); suction was then applied and the material again pressed down to a hard pad. This pad was broken with a spatula and transferred to a cylindrical separating funnel. The Büchner funnel and hardened paper were then well washed with water, the washings being transferred to the separating funnel to give a final aqueous volume of about 1500 ml. 100 ml. conc. HCl were then added and the mixture shaken vigorously to give a fine cream. To remove the P.T.A. the following organic solvent was used: amyl alcohol (pyridine-free and well washed with approximately *N* HCl), 20 parts; 0·720 ether, 25 parts; absolute ethanol, 1 part. 200 ml. of this were added to the funnel and the mixture shaken; a further 2 lots of 100 ml., making 400 ml. in all, were required to effect a clean separation. The solvent was drawn off and washed with 400 ml. of ether-water. This aqueous washing was added to the main aqueous solution, which was then washed with 4 lots of 50 ml. and 5 of 80 ml. of the organic solvent successively. The aqueous solution was then reduced *in vacuo* to a syrup to remove excess HCl. All the organic solvent solutions were then collected (vol. about 900 ml.), 600 ml. of ether added (to depress solubility of amino-acids) and the mixture washed 10 times successively with 60–70 ml. *N* HCl saturated with ether. The aqueous washings were collected (vol. about 1·5 l.) and washed 8 times successively with 60 ml. of the organic solvent. The latter were collected

(vol. about 450 ml.), 150 ml. ether added and the mixture washed 8 times successively with 70 ml. of the *N* HCl-ether-water. The collected aqueous washings were given two final washings with 40 ml. of the organic solvent and then reduced *in vacuo* to a syrup to remove excess HCl. This syrup, together with that from the main solution, was taken up in about 300 ml. water, filtered to remove a faint mist of amyl alcohol and the clear but slightly brown filtrate made up to 1 l. The total N was 3·894 g.≡33·8% T.P.N. The P.T.A. organic solvent solutions were reduced in volume to about 400 ml. and made up to 500 ml.; the total N was 6·7 mg.

11. The analysis of the base solution is described in Section 5.

12. The base filtrate was freed from P.T.A. by a procedure similar to that described above. The organic liquors retained only 6·0 mg. N. The clarified filtrate contained N≡34·9% and amino-N≡31·0% T.P.N. respectively. The procedure for the removal of inorganic ions was as follows.

Chloride. The excess HCl was removed by evaporation *in vacuo* to a gum, water being added and the operation repeated 6 times in all. The residue was taken up in water (300 ml.) and the bound HCl removed with moist Ag_2O, the solution being kept below *p*H 3 by the cautious addition of 0·5 *N* H_2SO_4, of which about 300 ml. were required. The AgCl was filtered off on a Büchner funnel, transferred to a beaker, brought to the boil with 700 ml. of water (stirring) and again filtered. The washing was twice repeated. The collected washings were reduced *in vacuo* to 150 ml. and allowed to stand; $CaSO_4$ separated and was filtered off. A small amount of $CaSO_4$ had also separated from the main solution on standing; this was also filtered off and joined to that mentioned above. The combined material was then washed 3 times with hot water and the washed $CaSO_4$ reserved. The AgCl was next washed 8 times in succession by bringing to the boil with about 300 ml. of *N* HCl and filtering. The washings were taken to dryness *in vacuo* to remove excess HCl, precipitated with Ag_2SO_4, the precipitate washed and the filtrate added to the main solution.

Silver. The solution and washings were treated with H_2S, the Ag_2S filtered off on a Büchner funnel and washed in the same way as the Cu_2S described above.

Calcium sulphate. The solution and washings were concentrated to the incipient crystallization of $CaSO_4$; 2 vol. of 95% ethanol were then added and the mixture allowed to stand overnight. The $CaSO_4$ was filtered off and washed 5 times successively by boiling with 100 ml. of water for 30 min. The washings were concentrated to about 60 ml. *in vacuo*, allowed to stand for a few hours, the small amount of separated $CaSO_4$ filtered off and washed 3 times with hot water.

Sulphate. The solution and washings were collected and sulphate precipitated as completely as possible with baryta, the mixture being heated on a water-bath for about 1 hr. The precipitate was filtered off on a Büchner funnel and washed 6 times successively by bringing to the boil with about 200 ml. of water. The filtrate and washings (at *p*H 7·3) were collected, reduced *in vacuo* to about 200 ml. and without removing the small amount of separated $BaSO_4$ a slight excess of Ba^{++} was carefully removed with H_2SO_4. The precipitate was filtered off and washed. A second and final adjustment for Ba^{++} and $SO_4^{=}$ was made after concentration of the filtrate and washings to a small volume.

The $BaSO_4$ and the AgCl from all these operations were collected and treated with 600 ml. *N* HCl at 80° for 8 hr. The filtrate and washings were collected, excess HCl and Cl^- removed in the usual way and the final filtrate added to the main solution. The working loss during the removal of all the above-mentioned inorganic ions was 17 mg. N actual and 23 mg. N corrected.

13, 14. In the edestin analysis stage 13 was omitted, and the solution of amino-acids was treated with $CuCO_3$ to make the Cu salts by the method of Town. These were granulated with A.R. acetone.

15, 16. (Abbreviation: M.A., monoamino-acids.) The dry salts, in a 250 ml. centrifuge bottle, were given 7 successive extractions with approximately 150 ml. carefully dried methanol (dehydrated twice over Ca) by shaking in a mechanical shaker for approximately 1 hr. each time. The insoluble residue was extracted repeatedly at room temperature with water until the extracts were colourless. These aqueous extracts were evaporated *in vacuo* and granulated with acetone as before. The dry material was then given 8 extractions as before with methanol. The residue from this last operation was taken up in water, evaporated to a syrup, and after granulation with acetone the extraction with methanol was continued, the 4th and 5th extracts being nearly colourless. The collected alcoholic extracts were reduced to a syrup, which was granulated with acetone and then re-extracted with the solvent. The alcoholic extracts were united and reduced to a syrup, which was then taken up in water and Cu^{++} removed with H_2S. After washing the CuS in the usual way the solution was reduced to 40 ml., and cooled to 0° overnight. The crystalline material that separated was filtered off, the mother liquor was reduced in volume, ethanol added to faint turbidity and the mixture warmed until the solution cleared. On standing at 0° overnight a 2nd crop of crystalline material separated. The collected products, M.A. crop 1, weighed 3·959 g. (5·318 g. corrected) and contained 11·74% N.

17, 18, 19. When present in fair concentration proline is partially precipitated by P.T.A. under the conditions of a base analysis; it was decided therefore to remove as much as possible of this imino-acid to prevent contamination of the second base precipitate at stage 25. The filtrate from the separation of M.A. crop 1 at stage 15 contained 0·165 g. amino-N and 0·204 g. non-amino-N (corrected 0·2216 and 0·2740 g. respectively). A small aliquot was first passed through the laborious $CdCl_2$ procedure of Town [1936], but the incidental losses were appreciable and the amount of non-amino-N in the final solution less than expected. Town himself found that the proline was not quantitatively precipitated by one treatment, and King [1941] records a similar finding. The remainder of the solution was therefore reduced to 30 ml. and picric acid (5·016 g.≡non-amino-N) in a small volume of hot water added. On standing at 0° a 1st crop of proline picrate (3·296 g.) separated and on reducing the volume a 2nd crop (1·153 g.) was obtained. The combined crops were equivalent to 1·996 g. proline (corrected). The mother liquor was acidified with HCl, the precipitated picric acid filtered off, the liquors extracted 6 times with ether to complete the removal, and reserved for stage 22.

20. Cu^{++} was removed from the solution of water-soluble salts, which was then reduced *in vacuo* to 25 ml. and kept overnight at 0°. M.A. crop 2 separated; wt. 1·9213 g. or 2·575 g. corrected; N=10·11%, all in the amino-form. The mother liquor was reserved for stage 22.

21. The residual Cu salts were extracted repeatedly with large volumes of water in a beaker heated on the water-bath, conditions which would remove any Cu aspartate. Each extract was filtered off, and while still hot Cu^{++} was

removed with H_2S. The CuS was washed in the usual way and the collected liquors reduced *in vacuo*. Three crops of monoamino-acids were obtained; collected these gave M.A. crop 3; wt. 7·3916 g. (corrected); N = 11·26 %. The mother liquor was reserved for stage 22. The final insoluble residue, which was thought at the time to be excess $CuCO_3$, almost certainly contained, according to later experience, the very insoluble Cu salt of methionine and possibly also that of phenylalanine; it also contained subordinate amounts of humin, and these products are in large part responsible for the recorded loss of N during the Cu salt treatment (stages 15–20), which amounted to 0·209 g. (corrected) ≡ 1·94 % T.P.N. To guard against the possibility that the 3 crops of monoamino-acids separated at stages 16, 20 and 21 respectively contained either aspartic or glutamic acid, though none gave any significant titration for dicarboxylic acids, an aliquot of each crop was taken, dissolved in hot water, NH_3 added to *p*H 8·8 and the mixture cooled [Osborne & Liddle, 1910*a*]. The crystalline mass of monoamino-acids was filtered off and the mother liquor reduced to a small volume. The lime-ethanol treatment was then applied, but the precipitate was free from N.

22, 23. (Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate.) The collected solutions were treated with a 1/10 vol. conc. HCl and boiled gently for 2 hr. to open the pyrrolidone ring. The subsequent treatment was similar to that applied at stages 7 and 8 and calls for no special comment. The lime-ethanol treatment was applied 3 times in all, from aqueous volumes of 60, 20 and 100 ml. respectively. The clarified solution from the 1st precipitate contained 0·1935 g. N (corrected 0·2599 ≡ 2·22 % T.P.N.) and 0·1538 g. amino-N; from it was isolated 0·5457 g. Cu-A and 0·4578 g. G-HCl. The 2nd treatment gave 0·1148 g. Cu-A and in 2 small crops 0·1609 g. G-HCl; the 3rd treatment 0·0848 g. G-HCl. The various lots were collected to give 0·9102 g. (corrected) of Cu-A (Table 2, crop 4) and 0·9684 g. (corrected) of G-HCl (Table 2, crop 5); the latter contained 4·96 % of $CaSO_4$. The final mother liquor contained 0·0270 g. N (corrected), equivalent to only 0·27 % T.P.N.

24, 25, 26. The procedure followed that of stages 10 and 11, the base precipitation being made from a total volume of 290 ml. with 25 g. P.T.A. in 20 ml. water. The base solution contained 0·1185 g. N of which 0·0778 g. was amino-N; the total N (corrected) was 0·1592 g. or 1·29 % T.P.N. The analysis for the 3 bases is given in Section 5; only 0·051 g. N, all in the amino-form and equivalent to 0·45 % T.P.N., was unaccounted for. The separation of proline at stage 18 had therefore been effective.

27, 28, 29. The base filtrate was freed from P.T.A. and all inorganic ions as in stages 10 and 12. The final solution was evaporated to a small volume and taken to dryness in a vacuum desiccator. The residue was a non-hygroscopic white powder; ash free it weighed 12·08 g. (corrected) and contained 13·15 % N. Tests for the presence of dicarboxylic acids and bases by the methods detailed more fully in the corresponding section of the egg-albumin analysis were all negative. The fraction will presumably contain all the non-nitrogenous products, except those condensed to humin, which had been formed by deamination of certain of the amino-acids during the 20 % HCl hydrolysis, evidence for which is revealed in the production of NH_3 in excess of the true amide-N.

Results of analysis

If present in the hydrolysate, tyrosine will be partially precipitated during the lime-ethanol treatment, so will phenylalanine and leucine if baryta be employed instead of lime. All three of these amino-acids, especially the former, will separate readily as the hydrochloride in the presence of 20 % HCl; they may, in consequence, be serious contaminants of the glutamic acid hydrochloride isolated at stages 8 and 23. In the procedure we have adopted the only likely contaminant is tyrosine hydrochloride, and as this is fairly readily soluble in absolute ethanol we always make a point of thoroughly washing our products with this solvent. The various crops of glutamic acid hydrochloride cited in Table 2 were analysed for ash and total N [Chibnall *et al.*, 1943], and their purity was controlled by confirming that the titration value in the micro-Kjeldahl was exactly one-half of that obtained when the same weight of material in aqueous solution was titrated to *p*H 7·3 (blue tint with bromothymol blue); working to fine limits, 1 % monoamino-acid impurity can be detected in this way. The necessary criteria for checking the purity of Cu aspartate have been discussed in a previous paper [Bailey *et al.*, 1943]: data for the various crops obtained in the present analysis are given in Table 2.

The detailed results of the complete analysis are given in Table 3. It is to be remembered that the

Table 2. *Analytical data for glutamic and aspartic acids from edestin*

	Glutamic acid hydrochloride			Copper aspartate			
						On anhydrous material	
Crops	Wt. (corrected) g.	N %	Rotation of glutamic acid in 9 % HCl $[\alpha]_D$	Wt. (corrected) g.	H_2O %	Cu %	N %
1	10·722	7·63	+30·3	10·71	29·15	32·54	7·22
2	1·994	7·65	+23·4	2·192	29·35	32·70	7·22
3	0·590	7·67		1·354	29·02	32·43	7·21
4	1·511	7·61	+ 7·3	0·910	29·36	32·28	7·11
5	0·928	7·68					
	15·745			15·166			
Theory		7·63			29·4	32·69	7·19

Table 3. *Analysis of edestin*

Stage (diagram 1)	Fraction	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
4	Mercaptide precipitates:				
	Humin	0·729	1·16	0·0759	0·61
	Cystine	0·846	1·37	0·0985	0·85
	Methionine and tyrosine	0·891	1·44	0·0796	0·74
6	Crude tyrosine	2·549	4·13	0·1973	1·71
7, 22	Glutamic acid	12·658	20·50	1·203	10·44
7, 22	Aspartic acid	7·372	11·95	0·7765	6·74
2	Ammonia	1·44	2·36	1·205	10·42
11, 23	Arginine	10·31	16·69	3·320	28·81
11, 23	Histidine	1·464	2·39	0·3948	3·47
11, 23	Lysine	1·431	2·32	0·2802	2·39
16	Monoamino-acids, crop 1 (N = 11·74 %)	5·318	8·61	0·6243	5·43
20	Monoamino-acids, crop 2 (N = 10·11 %)	2·575	4·17	0·2603	2·26
21	Monoamino-acids, crop 3 (N = 11·26 %)	7·606	12·32	0·8282	7·21
29	Monoamino-acids, crop 4 (N = 13·15 %)	12·22	19·56	1·59	13·86
18	Proline	1·996	3·23	0·2428	2·11
	Total accounted for	69·41	112·22	11·18	97·05
	Unaccounted for	3·27(?)	5·31	0·34	2·95
	Taken for analysis (61·74 g. protein)	72·68	117·7	11·52	100·0

value for proline represents that actually isolated as picrate and is not to be regarded as maximal; Osborne & Liddle [1910*b*] found 4·1 %.

The overall loss amounted to 2·95 % T.P.N., and the individual losses at the various stages are recorded in Table 4. The major loss occurs during the Cu salt treatment covering stages 14–21, and there is reason to believe that this falls almost exclusively on the more insoluble monoamino-acids, particularly methionine, and subordinate amounts of humin. The overall loss to stage 8, which occurred

Table 4. *Losses recorded at certain stages of analysis (edestin)*

Stages	Origin	Total N (corrected) g.	Total N (corrected) % total protein-N
1– 8	Overall losses to the removal of the main bulk of glutamic and aspartic acids	0·0304	0·26
11, 12	Retained by organic wash liquors	0·0128	0·11
12	Removal of inorganic reagents	0·0230	0·20
14–21	Copper salt fractionation; loss chiefly monoamino-acids	0·20	1·76
26	Unaccounted for in base solution	0·0519	0·45
21–29	Overall loss (diff.)	0·0219	0·22
1–29	Total	0·34	3·0

in large part during the working at that stage itself, must fall in part on the dicarboxylic acids, but from stage 9 onwards the concentration of these, especially at stage 25, is so low relative to the other products present that their contribution to the remaining losses must be very small indeed. In a previous paper [Bailey *et al.*, 1943] we have discussed the solubility of the Ca salts of the dicarboxylic acids in the lime-ethanol procedure, and have given our reasons for the belief that in the present edestin analysis small solubility factors can be legitimately applied at stage 22. When account has been taken of these factors our final results for glutamic acid and aspartic acid are 20·70 and 12·0 % W/P respectively (Table 14), and in view of what has been said above we believe that these are within 1–2 % of the true values. Solubility corrections for the bases are discussed in Section 5.

Reverting to the data given in Table 3, we see that 69·41 g. of hydrolysis products have been identified, and that these represent 97·05 % of the total protein-N. If we assumed for reasons given above that the loss to stage 8 represents dicarboxylic acids with an average of 10 % N, that the loss covering stages 14–21 represents monoamino-acids with an average of 9 % N, and that the other losses represent amino-acids with an average of 12 % N, then calculation shows that the gross weight of unidentified products, containing 0·34 g. N, would be 3·27 g. This value has been incorporated in Table 3 and permits the deduction that 100 g. protein have given 117·7 g. hydrolysis products. The computed value, based on the total N and the peptide-N of the protein [Chibnall, 1942], is 117·8; agreement is good, considering the assumption made with respect to the humin and unidentified products in Table 3.

3. *Analysis of egg albumin*

The edestin procedure needed slight modification on account of the larger proportion of monoamino-acids in the egg albumin hydrolysate. In the description that follows only points of difference or of unusual interest will be noted.

Details of analysis

1, 2. The sample of coagulated protein [Chibnall *et al.*, 1943] contained 0·44 % ash, and ash- and moisture-free

15·76% N. 62·02 g. ash- and moisture-free protein containing 9·78 g. N were dissolved in 600 ml. 20% HCl by heating on a water-bath for 6 hr. The mixture was then boiled under reflux for a further 24 hr. and the excess HCl removed in the usual way. Acid humin was then removed and washed 3 times with cold and 3 times with hot water; it contained 7% ash. The ash-free product weighed 0·862 g. and contained 0·0633 g. N. To avoid the withdrawal of a large aliquot from the main hydrolysate to determine alkali humin, part of which is carried down in the mercaptide precipitate, a separate small hydrolysis under identical conditions was carried out for this purpose. Alkali humin was determined by adding CaO to *p*H 7·5. The weight, ash-free, and calculated in terms of the main hydrolysate, was 1·061 g. and the total N 0·0652 g. The filtrate was deep straw-coloured, showing that all the humin had not been removed.

3, 4, 5. As 0·0162 g. of alkali humin-N was removed in the lime-ethanol treatment at stage 8 it is assumed that the remainder of that estimated above has been carried down in the mercaptide precipitate. The latter (wt. 50·5 g.) contained organic-S in excess of that due to cystine and by difference 0·3% w/P of tyrosine. It is clear, therefore, that as in the case of edestin the conditions during the precipitation have caused the separation of the less soluble monoamino-acids or their copper complexes. To ascertain whether aspartic acid or arginine were concerned, 10 g. of the product was dissolved in 250 ml. HCl, Cu^+ removed with H_2S, the precipitate of Cu_2S and humin well washed and the clarified solution reduced *in vacuo* to remove excess HCl. Treatment with flavianic acid by the method of Vickery [1940] gave 21·4 mg. recrystallized material [Lucas & Beveridge, 1940] which was definitely not arginine monoflavianate, for it did not possess the characteristic golden lustre and on attempting a second recrystallization (see Section 5) it remained in solution. The flavianate mother liquors were collected, excess flavianic acid removed as described in Section 5, and the lime-ethanol treatment for dicarboxylic acids applied from an aqueous volume of 18 ml. The Ca precipitate, when treated as in stage 8, gave a solution containing 42·2 mg. N and from this various crops of impure Cu cysteate were separated, but a careful search failed to give evidence for the presence of Cu aspartate. The final data for the composition of the mercaptide precipitate are given in Table 5. The mercaptide filtrate, still somewhat coloured, was freed from Cu^+ with H_2S without the addition of HCl, consequently the concentration of Cl^- at the 1st lime-ethanol stage was very low.

Table 5. *Composition of the organic material in the mercaptide precipitate (egg albumin)*

	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
Alkali humin	0·74	1·20	0·0490	0·50
Cystine	1·10	1·78	0·1282	1·31
Methionine	1·60	2·59	0·1582	1·62
Monoamino-acids (diff.)	0·59	0·95	0·0589	0·60
	4·03	6·52	0·3943	4·03

7, 8, 9. Two successive treatments with lime-ethanol were made from aqueous volumes of 500 ml. There was insufficient Cl^- present at the second operation to flocculate the precipitate, and it was necessary to add 8 g. crystalline $CaCl_2$. On redissolving the precipitate the residual lime retained 0·0162 g. alkali humin-N. During the treatment with HCl to effect the separation of G-HCl much darkening of the solution occurred, due presumably to the decomposition of the glucosamine moiety of the polysaccharide present in egg albumin [cf. Neuberger, 1938]. As a result 0·157 g. acid humin, containing 0·0122 g. N, was removed prior to the 3rd treatment with lime-ethanol, which was made from an aqueous volume of 500 ml. The 4th treatment was also made from a similar volume, and in the final clearing of the material recovered from the precipitate it was necessary to remove $SO_4^=$ (from the protein ash) as $CaSO_4$ with ethanol. From all these operations 4 crops of Cu-A, containing aspartic acid equivalent to 7·74% w/P, and 4 crops of G-HCl, containing glutamic acid equivalent to 13·89% w/P, were obtained. The final filtrate contained (corrected) 0·0932 g. N of which 0·0664 g. (≡0·68% T.P.N.) was amino-N. The overall loss to the end of stage 8 was 14·7 mg. N (17·9 mg. N corrected) and the flow sheets showed that this occurred in large part during the separation of the glutamic and aspartic acids.

9, 10, 11. The precipitation of the bases with P.T.A. was made from a total volume of 2 l. As the hydrolysate contained such a high proportion of monoamino-acids the base solution, freed from P.T.A., was reprecipitated from a total volume of 1450 ml., the mixture being allowed to stand at 0° overnight. It is doubtful if any benefit resulted from taking this precaution, as the residual solubility of the arginine phosphotungstate was considerable: the final base solution nevertheless was free from extraneous-N (see Section 5). The whole operation, including the removal of P.T.A. from the two filtrates, resulted in a loss of only 8·7 mg. N (11·1 mg. N corrected).

The loss on removing all inorganic reagents and excess Cl^- was 7·4 mg. N (10·1 mg. corrected). On evaporation of the clarified solution 3 crops of monoamino-acids were removed.

14–21. The filtrate from stage 13 was brownish, and clearly contained humin, part of which probably may have resulted from the operations of stage 8. On treating the solution with excess $CuCO_3$ to make the Cu salts a small reduction to Cu_2O occurred, presumably due to glucosamine or its derivatives. The extraction of the dry salts with ethanol and cold water proceeded smoothly and but little material was subsequently removed from the residue by treatment with boiling water, probably because the amino-acids concerned had been separated at stage 13. On removal of Cu^{++} from the various fractions the CuS retained 50 mg. humin-N. The separation of proline, at the picric acid stage, was more difficult than in the case of edestin on account of the presence of hydroxyproline, and fractional crystallization was necessary. The yields of the two picrates (m.p. 153° and 189°) were equivalent to 1·632 g. of proline or 2·63% w/P and 0·262 g. hydroxyproline or 0·42% w/P respectively. The overall loss in the complete Cu treatment was 0·144 g.; as in the case of edestin we believe the major part of this to be due to the insoluble Cu salt of methionine.

22, 23. The collected solutions were treated with a 1/10 vol. conc. HCl and gently boiled for 2 hr. to convert any pyrrolidone carboxylic acid present to glutamic acid. Excess HCl was then removed and the lime-ethanol treatment applied 3 times in succession from 200 ml. of aqueous solution. The clarified solution from the ultimate precipitate contained 0·2057 g. N (0·385 g. corrected) of which only 0·147 g. was amino-N, indicating the presence of much

arginine due to recrystallization of the base precipitate at stage 10. G-HCl crop 5, and a fraction of Cu-A were separated: the latter needed recrystallization. The lime-ethanol treatment was then applied once more from 200 ml.; the clarified solution from the precipitate contained 0·0807 g. N and 0·0597 g. amino-N. G-HCl crop 6, and a fraction of Cu-A were separated; the latter was added to that mentioned above to give Cu-A crop 5. The final mother liquor contained 58 mg. of amino-N (corrected) equivalent to 0·59% T.P.N. Much of this was probably due to serine, as egg albumin contains many per cent of this hydroxy-amino-acid [Martin & Synge, 1941]. The loss during these operations was 18·7 mg. (corrected), part of which must fall on the dicarboxylic acids.

24, 25, 26. The base precipitation was made from a total volume of 290 ml. The precipitate was filtered off, washed on the filter paper and then redissolved (incompletely) by warming with 180 ml. of P.T.A. wash solution for 10 min. at 80°. The mixture was allowed to stand at room temperature for 2 hr. and then filtered off. The filtrate was shown later to contain much arginine, and as in stage 10, it is doubtful if this recrystallization was of any advantage.

27, 28, 29. The clarified solution on evaporation to a small volume gave M.A. crop 5, containing 12·31% N and on taking the mother liquor to dryness in a vacuum desiccator there was obtained a final fraction containing 3·7% ash. Ash-free, this residual product weighed 4·34 g. (9·297 g. corrected).

As the base precipitate at stage 25 had been recrystallized we suspected that a small amount of arginine phosphotungstate had remained in solution. Accordingly 2·604 g. of the final residue was dissolved in 75 ml. water and arginine flavianate precipitated by the method of Vickery [1940], using 2·4 g. flavianic acid. The yield of arginine flavianate (tested for purity as described in Section 5) was equivalent to 0·1358 g. arginine (corrected). The excess flavianic acid was removed from the filtrate by strongly acidifying with HCl and extracting with amyl alcohol-ether mixture. The aqueous solution freed from excess HCl was then submitted to the lime-ethanol treatment from a volume of 50 ml. The clarified solution from the precipitate contained 38·7 mg. N, equivalent to 0·88% T.P.N. and it was considered that the major part of this would be due to serine. Abderhalden & Kautzsch [1912] observed that aspartic and glutamic acids were precipitated by Hg acetate, and we have found that this is so even in the presence of 10 times the equivalent of serine. The above-mentioned solution was treated accordingly and the precipitate freed from Hg^{++} with H_2S. The clarified solution (8·9 mg. N≡0·3% T.P.N.) gave a small amount of good crystallin Cu aspartate, in amount (corrected) equivalent to only 17 mg. of aspartic acid or 0·03% W/P and in excellent agreement with the calculated solubility correction [Bailey *et al.*, 1943] for stage 23. No glutamic acid hydrochloride could be isolated.

The mother liquors and the various filtrates were then collected, all reagents removed, and the clarified solution submitted to electrodialysis by Mr A. H. Gordon. Only 3 mg. N (10·6 mg. N corrected≡0·11% T.P.N.) passed into the cathode compartment, showing that no appreciable basic-N was present. The object of this last experiment was to demonstrate the absence of ornithine, for the properties of this base are such that it would almost certainly have escaped precipitation with phosphotungstic acid at stages 10 and 25. In Table 7 monoamino-acids crop 7 represents the residual 9·297 g. less the amount of arginine and aspartic acid subsequently isolated.

Results of analysis

The analytical data for the various crops of glutamic acid hydrochloride and copper aspartate are given in Table 6. Calculation shows that the glutamic acid hydrochloride isolated at stage 8 (i.e. crops 1–4) represents 8·269 g. of *l*(+)-glutamic acid and 0·384 g. of *dl*-glutamic acid; the corresponding weights for stage 23 are 0·721 and 0·6 g. respectively, showing that the solubilities of the two Ca salts in the modified Foreman procedure are similar [Bailey *et al.*, 1943] and refuting the contrary claim of Kögl, Erzleben & Akkermann [1939]. 9·8% of the total glutamic acid isolated at stages 8 and 23 can be regarded as fully racemized.

The detailed results of the analysis are given in Table 7. The main working losses have been indicated in the text and if these are evaluated as in the case of edestin it is possible to suggest the data given in the table for the products unaccounted for. The weight of hydrolysis products given by 100 g. protein is 117·8 g., a value that cannot be accurate to ±0·5 g. on account of the difficulty of assessing the alkali humin fractions, into which, with the acid humin, most of the mannose and glucosamine of the polysaccharide moiety must have passed through condensation with tryptophan and subordinate amounts of cystine and tyrosine [Lugg; see Chibnall, 1939, p. 162]. The computed value is 117·2 g. [Chibnall, 1942]. When account is taken of the small solubility corrections applicable at stage 22

Table 6. *Analytical data for glutamic and aspartic acids from egg albumin*

	Glutamic acid hydrochloride			Copper aspartate			
						On anhydrous material	
Crops	Wt. (corrected) g.	N %	Rotation of glutamic acid in 9% HCl $[\alpha]_D$	Wt. (corrected) g.	H_2O %	Cu %	N %
1	7·425	7·65	+31·7	6·319	29·14	32·5	7·15
2	1·762	7·65	+31·3	2·272	29·32	32·5	7·20
3	0·735	7·64	+29·7	0·845	29·3	32·5	7·19
4	0·770	7·61	+13·6	0·479	28·6	32·6	7·15
5	0·884	7·67	+26·1	0·459	29·2	32·4	7·18
6	0·727	7·66	+ 5·5	—	—	—	—

Table 7. *Analysis of egg albumin*

Stage (diagram 1)	Fraction	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
2, 8	Acid humin	1·019	1·64	0·0755	0·77
3, 8, 20, 21	Alkali humin	2·110	3·40	0·1344	1·37
4	Mercaptide precipitates:				
	Cystine	1·100	1·77	0·1282	1·31
	Other amino-acids	2·190	3·54	0·2171	2·22
6	Crude tyrosine	1·511	2·44	0·1184	1·21
2, 8, 26	Ammonia	1·071	1·73	0·8909	9·11
7, 22	Glutamic acid	9·913	16·00	0·9428	9·64
7, 22	Aspartic acid	5·019	8·10	0·5294	5·41
11, 23, 29	Arginine	3·483	5·62	1·1211	11·46
11, 23	Histidine	0·901	1·45	0·2443	2·50
11, 23	Lysine	3·136	5·06	0·6010	6·15
13	Monoamino-acids, crop 1 (N = 10·71 %)	10·120	16·32	1·0830	11·08
13	Monoamino-acids, crop 2 (N = 11·04 %)	2·557	4·12	0·2822	2·89
13	Monoamino-acids, crop 3 (N = 11·35 %)	5·443	8·78	0·6179	6·32
16	Monoamino-acids, crop 4 (N = 10·88 %)	3·466	5·59	0·3772	3·86
16	Monoamino-acids, crop 5 (N = 10·85 %)	2·589	4·18	0·2826	2·89
28	Monoamino-acids, crop 6 (N = 12·31 %)	3·624	5·84	0·4650	4·76
28	Monoamino-acids, crop 7 (N = 12·25 %)	9·142	14·74	1·1381	11·64
18	Proline	1·632	2·63	0·1920	1·96
19	Hydroxyproline	0·262	0·42	0·0383	0·40
1–29	Total accounted for	70·29	113·35	9·50	96·95
	Unaccounted for	2·55(?)	4·13	0·28	2·86
	Taken for analysis (62·02 g. protein)	72·84	117·5	9·78	100·0

the final results for glutamic acid and aspartic acid are 16·1 and 8·13 % w/p respectively (Table 14). As the gross losses at stages 8 and 23 totalled only 36·6 mg. N we believe that these results are within 1–2 % of the true values.

Throughout the analysis we have kept in mind Fearon's [1939] recent claim that egg albumin contains citrulline. The unhydrolysed protein gave the bright purple-red colour with diacetyl monoxime, as he states. After acid hydrolysis the colour was redder, yet it did not match the characteristic red given by citrulline itself; the colour moreover faded rapidly, whereas that given by citrulline is stable for many days. This last difference became more pronounced as the hydrolysate was fractionated, and the various crops of monoamino-acids described in the text and in Table 7 failed to give the test at all. Citrulline is not very stable on acid hydrolysis, as shown by the fact that a sample of 0·2 g., when boiled with 10 ml. of 20 % HCl for 22 hr., yielded 14·2 % of the total N as amide-N; even so we should have expected to find evidence for it in the amino-acid mixtures recovered from the Cu salts at stages 16, 20 or 21 if any appreciable amount had been present in the hydrolysate. Our evidence therefore does not support Fearon's claim.

4. *Analysis of β-lactoglobulin*

In light of the experience gained with the other two proteins the procedure used in this case was considerably simplified.

Details of analysis

1, 2. 30·61 g. moisture- and ash-free coagulated protein, containing 4·76 g. N or 15·55 %, were heated on the water-bath with 250 ml. 20 % HCl until solution was effected and then boiled under reflux, for an inclusive period of 24 hr.

4. The mercaptide precipitated was analysed for N but not for S or for humin.

7, 8, 9. Three lime-ethanol treatments were made in succession from an aqueous volume of 250 ml. before the separation of the dicarboxylic acid derivatives was attempted. The clarified precipitate contained 1·042 g. N (corrected) ≡ 21·9 % T.P.N. Repeat precipitations were made in due course from 200 and 100 ml. respectively.

9, 10, 11. The base precipitation was made from a total volume of 1600 ml. The total N in the precipitate was 0·9282 g. (corrected) (see Section 5).

13. Four crops of monoamino-acids were obtained, weighing 4·062, 1·360, 2·951 and 1·557 g. and containing 10·07, 10·67, 11·02 and 11·81 % N respectively.

14–21. The Cu salt procedure was omitted, as a determination of amino-N on the filtrate from 13 indicated that the proline and hydroxyproline content must be fairly small.

22, 23, 24. The lime-ethanol treatments were made in succession from aqueous volumes of 100, 200 and 200 ml. respectively before the separation of the dicarboxylic acid derivatives was attempted. The clarified precipitate contained 0·0708 g. N ≡ 1·49 % T.P.N. A final treatment from 50 ml. was made in due course. The ultimate mother liquor contained 13·3 mg. N (corrected), equivalent to only 0·28 % T.P.N.

25, 26. The 2nd base precipitation was made from a total volume of 265 ml. The total N in the precipitate was 54·2 mg. all of which was accounted for as base (see Section 5).

28, 29. Two crops of amino-acids and a final residue were obtained; these weighed 1·745, 3·59 and (ash not deducted) 5·94 g. and contained 11·37, 12·38 and 10·5 % N respectively. The latter product was treated with flavianic acid, but no arginine salt could be separated.

Results of analysis

The data given in Table 8 summarize the fractionation of the N; as the mercaptide precipitate was not examined in detail it is not possible to summarize the hydrolysis products as in the case of the other two proteins. The overall loss was 59·9 mg. N (corrected), while the losses on working stages 8 and 23 were 5·0 and 1·6 mg. N respectively, jointly equivalent to 0·14 % T.P.N., part of which must fall on the dicarboxylic acids. The final values for arginine, histidine, lysine, glutamic acid and aspartic acid are 2·89, 1·54, 9·75, 21·32 and 9·83 % W/P respectively. If the small solubility corrections for stage 22 be applied [Bailey *et al.*, 1943] the two latter values are raised to 21·51 and 9·88 % respectively (Table 14) and we believe these are accurate to 1–2 %. The solubility corrections for the bases are discussed in Section 5.

Table 8. *Analysis of β-lactoglobulin*

Stage (diagram 1)	Fraction		N g.	N as % total protein-N
2	Ammonia		0·3593	7·55
3	Mercaptide precipitate		0·2090	4·39
8	Glutamic acid	0·5982 g.	0·6199	13·03
23	Glutamic acid	0·0217 g.		
8	Aspartic acid	0·2912 g.	0·3159	6·65
23	Aspartic acid	0·0247 g.		
8	Crude tyrosine		0·0220	0·46
11	Arginine	0·2623 g.	0·2799	5·88
26	Arginine	0·0176 g.		
11	Histidine	0·1076 g.	0·1247	2·62
26	Histidine	0·0171 g.		
11	Lysine	0·5537 g.	0·5719	12·01
26	Lysine	0·0182 g.		
13	Monoamino-acids in 4 crops		1·0637	22·35
28, 29	Monoamino-acids in 3 crops		1·1332	23·81
	Total accounted for		4·6995	98·75
1, 29	Unaccounted for		0·0599	1·25
	Taken for analysis		4·76	100·0

5. *Analysis of the base fractions*

The present study is to be regarded as a continuation of that of Tristram [1939] on the reliability of various methods for estimating the basic amino-acids in protein hydrolysates. Tristram favoured the use, with certain minor modifications, of Block's [1938] small-scale adaptation of the modified Kössel procedure devised by Vickery and his co-workers [Vickery & Leavenworth, 1928; Vickery & Shore, 1932; and Vickery & White, 1933] and he tested this, *inter alia*, against appropriate mixtures of the bases representing 2–3 g. of protein. The losses at the various stages in the analysis were determined. Having allowed for the solubility of arginine silver [1·1 mg. N/100 ml., Gulewitsch, 1899], the overall loss in the estimation of arginine was 2·6 mg. N, due in part to precipitation with the histidine silver at *p*H 7·2 and in part to incomplete precipitation as arginine silver at *p*H 12–13. The overall loss in the estimation of histidine was small (0·3 mg. N/100 ml.) while that for lysine (1·6 mg. N/100 ml.) was but little more than that due to the solubility of lysine phosphotungstate. Tristram considered that corrections to allow for these overall losses could be legitimately applied when analysing a mixture of the three bases, but that in the case of protein hydrolysates the validity of such corrections must remain a matter of conjecture until new methods of analysis were found which would permit a direct determination of the solubility factors concerned. Examination of the base solutions obtained during the fractionation of the hydrolysates described in Sections 2–4 has enabled us to investigate these solubility factors in detail.

Base fraction of β-lactoglobulin

The two base fractions described in Section 4 were analysed by the Tristram-Block procedure mentioned above, the only modifications being the employment of Block's [1940] nitranilic acid method for the estimation of histidine and the thorough washing of all inorganic precipitates as described in Section 2. The purity of the arginine flavianate, which might be contaminated with the histidine salt, was controlled by recrystallization under standard conditions detailed later in our discussion of Vickery's method for arginine. Arginine nitranilate is fairly soluble under Block's conditions, but the lysine salt is as insoluble as that of histidine itself. As nitranilic acid is readily decomposed in hot water [Town, 1936] the purity of the isolated histidine salt was controlled in the following way. 0·1 g. material and 25 ml. water were placed in a 100 ml. beaker, which was then partly immersed for exactly 4 min. in a water-bath at 65°. Solution of the salt is readily effected on stirring, and if there be an insoluble residue the presence of Ba nitranilate may be suspected. The beaker was then removed from the bath, 25 ml. of methanol were run in with stirring, and crystallization allowed to proceed for 2 days at 2°. The filtered, washed and dried product should weigh 0·0980 g. Under the conditions of a base analysis it is improbable that lysine would pass into the histidine fraction, but the procedure serves to detect the presence of the Ba salt, a possible contaminant.

Analysis of 1st base solution. The aliquot used contained 0·3664 g. N; as this amount of basic-N was approximately 3 times that present in the hydrolysate of 2–3 g. protein the separation into the 3 base fractions was made from 3 times the respective volumes employed by Tristram. Excess HCl was removed by evaporation *in vacuo*, and the solution freed from Cl^- with Ag_2O and H_2SO_4 at *p*H 3·0

in the usual way. The arginine, histidine and lysine fractions contained 0·1034, 0·0394 and 0·2207 g. of N respectively. The N recovered in the form of base derivatives in this first analysis is given in Table 9, and allowing solubility corrections (300 ml.) for lysine phosphotungstate and arginine silver the N unaccounted for was 9·5 mg. The mother liquors of the 3 derivatives were collected, reduced *in vacuo* to a few ml., strongly acidified with HCl and the 3 acidic reagents extracted by the amyl alcohol-ether procedure for the removal of excess phosphotungstic acid, an operation that can be conducted without loss of basic-N. The clarified solution, freed from HCl and Cl^- and containing 7·0 mg. N, was then passed once again through the Tristram-Block procedure, using a third of Tristram's volumes. The weight

Table 9. *Analysis of the base fractions of β-lactoglobulin*

	1st base solution		2nd base solution		
	N g.	N (corrected) as % total protein-N	N g.	N (corrected) as % total protein-N	Total base N as % total protein-N
Aliquot taken for analysis	0·3664	19·50	0·0496	1·10	20·60
Arginine isolated as flavianate, 1st analysis	0·0975	—	0·0135	—	(5·43)
Correction for solubility of arginine silver, 1st analysis	0·0033	—	0·0011	—	—
Correction for overall losses, 1st analysis	0·0026	—	0·0026	—	—
Total arginine	0·1034	5·51	0·0172	0·37	5·88
Histidine isolated as nitranilate, 1st analysis	0·0375	—	0·0162	—	—
Histidine isolated as nitranilate, 2nd analysis	0·0048	—	—	—	—
Total histidine	0·0423	2·26	0·0162	0·36	2·62
Lysine isolated as picrate, 1st analysis	0·2170	(11·53)	0·0159	—	(11·88)
Correction for solubility of lysine phosphotungstate	0·0015	—	0·0008	—	—
Total lysine	0·2185	11·63	0·0166	0·37	12·0
Total base accounted for	0·3642	19·38	0·50	1·11	20·49

of histidine nitranilate obtained in the 2nd analysis was 40·8 or 43·8 mg. corrected to the 1st base solution, equivalent to 4·8 mg. histidine-N. This amount of histidine must have passed into the arginine fraction in the 1st analysis. No flavianate was obtained from the arginine fraction of the 2nd analysis. The complete results for the 1st base solution are set out in Table 9. As it is clear that no appreciable amount of extraneous non-basic material is present we have included Tristram's correction of 2·6 mg. for overall losses of arginine-N, though we worked to 3 times his volumes; such an amount is, in fact, insufficient to lead to any recovery of arginine flavianate in the 2nd analysis.

The 2nd base solution was passed once through the Tristram-Block procedure, working to Tristram's volumes. Again allowing a correction of 2·6 mg. for overall losses of arginine-N the recovery of basic-N is complete, as shown in Table 9. It may be mentioned that in all the above-mentioned analyses the arginine flavianate and histidine nitranilate were shown to be pure by the solubility test mentioned earlier, and the lysine picrate, without recrystallization, exploded sharply at 266–267°. The overall correction for working losses applied to arginine accounts for 0·2% T.P.N. and this must be considered the one uncertainty in the full analysis.

It is of interest to consider the solubility of the phosphotungstates on precipitation of the bases. Relevant data are collected in Table 10. The volume employed in the 1st precipitation was 1600 ml. and in the 2nd 265 ml. On the assumption that the phosphotungstate solubilities in the two cases were proportional to the volumes employed, which may not be true as the amino-acid composition and concentration in the respective solutions was not the same, the amounts of the bases isolated from the 2nd base solution (column 1) represent the solubilities in 1335 ml. From these the solubility corrections for the 2nd and 1st precipitations (columns 2 and 3) have been calculated. The original Van Slyke corrections for the latter are given in column 4. Comparison is not strictly valid as we did not use P.T.A. purified by Winterstein's method, but our low value for histidine is what would be expected from the more recent work of Van Slyke *et al.* [1942]. The two arginine values show fair agreement, while the great difference between those for lysine suggests that the amino-acid mixture has influenced the solubility in our case. The final values for the bases given in Table 14 include the solubility corrections (Table 10, column 2) for the 2nd precipitation. We believe that these will be found to be within 2% of the true values.

Table 10. *Base phosphotungstate solubility corrections for β-lactoglobulin*

(Values quoted are in percentages of total protein-N.)

	Isolated from 2nd base solution	Solubility corrections		Van Slyke solubility corrections
		2nd base precipitation	1st base precipitation	1st base precipitation
	(1)	(2)	(3)	(4)
Arginine	0·37	0·07	0·44	0·51
Histidine	0·36	0·07	0·43	0·64
Lysine	0·37	0·07	0·44	0·08

Direct Tristram-Block analysis. 3·803 g. moisture- and ash-free protein, containing 0·5895 g. N, were hydrolysed with H_2SO_4 for 24 hr. The analysis followed the lines of those mentioned above, including the thorough washing of all inorganic precipitates. The arginine flavianate and histidine nitranilate were of satisfactory purity; the lysine picrate was recrystallized from 15 ml. water and corrected for solubility (3·4 mg. N/ml. at 0°); it exploded at 266–267°.

The collective results given in Table 11 bring out some interesting comparisons with Table 9. In the Tristram-

Table 11. *Tristram-Block analysis of β-lactoglobulin*

	Total N g.	N as % total protein-N
Arginine fraction	0·0394	
Arginine isolated as flavianate	0·0316	(5·36)
Correction for arginine silver (150 ml.)	0·0016	
Correction for overall losses	0·0026	
Total arginine	0·0358	6·07
Histidine fraction	0·1292	
Histidine isolated as nitranilate	0·0133	2·26
Lysine fraction	0·1186	
Lysine isolated as picrate	0·0681	(11·55)
Correction for overall losses	0·0016	
Total lysine	0·0697	11·82

Block analysis the arginine flavianate isolated was equivalent to 5·36% of the total protein-N, while our comprehensive procedure gave 5·43%; yet the corrected values were 6·07 and 5·95% respectively, suggesting that in the direct analysis the amount of N allowed for overall losses was a little too high. The histidine value in the Tristram-Block analysis is low and as according to our experience the total N in the histidine fraction varies enormously from protein to protein it is difficult to suggest any general overall corrections. With a mixture of bases Tristram's losses by his procedure were negligible (0·3 mg. N) and we confirm Block's finding that under the conditions that he applies nitranilic acid the precipitation of histidine in the absence of extraneous material is quantitative. Whether the low yield in the present case is to be ascribed to the increased solubility of histidine silver in the presence of the protein hydrolysis products or to that of histidine nitranilate in the presence of nearly 10 equivalents of extraneous N cannot be stated with certainty; both factors are probably operative, each to a degree that will vary from protein to protein. In the Tristram-Block analysis the lysine value based on the isolated picrate was 11·55%, in excellent agreement with the 11·53% obtained from the first phosphotungstate precipitate in the comprehensive procedure. The corrected value is a little low, as one would expect from our demonstration that the solubility of the phosphotungstate is enhanced by the presence of the amino-acids of the hydrolysate: even so the presence of much extraneous material in the lysine fraction has probably prevented the quantitative separation of the lysine as picrate in one operation [Vickery & Leavenworth, 1928].

Base fractions of edestin and egg albumin

The procedure used was the same as that described for lactoglobulin, and the results are given in Table 12. In the case of edestin the volume ratio in the two base precipitations was 9 to 1, so that the calculated corrections for the phosphotungstate solubilities in the 2nd were 0·04, 0·03 and 0·05% T.P.N. respectively. The final values for the bases are given in Table 14.

Conditions were different in the case of egg albumin, for both phosphotungstate precipitates were recrystallized. From the results given in Table 12 it would appear that on recrystallization of the 1st precipitate the arginine phosphotungstate separated as a gelatinous rather than a crystalline precipitate [Van Slyke *et al.*, 1942] and its solubility has been much enhanced. As the 2nd base precipitate was also recrystallized we inspected a similar occurrence and accordingly estimated the amount of arginine in the amino-acid fraction at stage 29 by the Vickery flavianic acid method discussed later. This fraction contained 12·1% of the T.P.N. and from it was isolated arginine equivalent to 0·41% of the T.P.N. After removal of the excess flavianic acid, and also of the residual dicarboxylic acids, from the arginine flavianate filtrate, the clarified solution gave on electrodialysis 0·11% T.P.N. in the cathode compartment. This value sets a definite limit to the residual solubilities of the 3 bases, as it will include any monoamino-acid-N which had migrated by simple diffusion. Our final results for egg albumin are therefore those given in Table 12, and are believed to be not more than 2% low.

Direct arginine analysis by the method of Vickery

In the comprehensive analyses mentioned above our original plan had been to effect a preliminary removal of arginine as flavianate by Vickery's [1940] new method so that the histidine and lysine fractions in the subsequent Tristram-Block procedure would be free from this base. The expectation was not realized, for in the absence of all the non-basic products of hydrolysis the recrystallized product was found to be heavily contaminated with the flavianates of the other two bases, and repeated recrystallization was necessary to obtain pure arginine flavianate. As this latter substance has an appreciable solubility under the conditions employed small amounts of it passed into the various mother liquors and washings, so that the incorporation of this additional step in the analysis of the base solutions defeated its own end. These findings prompted us to investigate the purity of the arginine flavianate obtained when the simple Vickery procedure for this base is applied directly to solutions containing known amounts of the three bases, and to protein hydrolysates.

Table 12. *Analysis of the base fractions of edestin and egg albumin*

	Edestin				Egg albumin			
	N as % of total protein-N				N as % of total protein-N			
	1st base solution	2nd base solution	Total	Wt. (% of protein)	1st base solution	2nd base solution	Total	Wt. (% of protein)
Arginine	28·47	0·33	28·80	16·69	8·20	2·85	11·46*	5·63
Histidine	3·20	0·27	3·47	2·39	2·27	0·23	2·50	1·45
Lysine	2·01	0·38	2·39	2·32	5·98	0·17	6·15	5·06

* Included 0·41% isolated as flavianate at a later stage than the 2nd base precipitation.

Precipitation of arginine as flavianate. 30 ml. of a solution of arginine dihydrochloride containing 0·2084 g. arginine were treated with 1·84 g. (5 mol.) flavianic acid according to Vickery's directions. After 4 days at 2° the precipitate was filtered off, washed with 30 ml. of water saturated at room temperature with arginine monoflavianate and recrystallized from 25 ml. via NH_3 and H_2SO_4. The precipitate weighed 0·5805 g. (theory 0·5845 g.) and on further recrystallization 5 times successively under the same conditions, the losses in weight were 3·0, 3·7, 2·2, 3·2 and 2·4 mg. respectively, or an overall mean of 3·0 mg. If this correction be applied to the weight of flavianate obtained after 1 recrystallization, giving a total of 0·5840 g. in all, the recovery is almost theoretical and confirms Vickery's claim that the diflavianate, in which form most of the arginine separates in the initial stage, is insoluble in the presence of an excess of flavianic acid. A similar experiment, using a volume of 50 ml. for the preliminary separation and for all subsequent recrystallizations, gave a mean overall loss of 4·0 mg.

Precipitation of arginine as flavianate from a mixture of bases. (1) The solution (30 ml.) contained 0·2084 g. arginine, 0·171 g. lysine and 0·0505 g. histidine, the relative proportions being approximately the same as in egg albumin. It was treated with 1·84 g. flavianic acid and the recrystallized product (from 25 ml.), which appeared to consist of plates with the golden yellow lustre characteristic of arginine monoflavianate, weighed 0·6465 g. The losses on successive recrystallization were 46·9, 15·8, 6·8, 7·9, 3·1, 2·2 and 3·1 mg. suggesting that 5 recrystallizations in all were necessary to give pure arginine monoflavianate. Applying the overall correction (8 × 3·0 mg.) the corrected weight was 0·5848 g. giving a theoretical estimation of the arginine present. (2) The solution (30 ml.) contained the same weight of arginine as before, and the other two bases in the relative proportions that occur in edestin. After 1 recrystallization the weight of the product was 0·595 g. and the losses in 2 subsequent recrystallizations were 19·2 and 3·0 mg. respectively. Applying the overall correction of 9·0 mg. the yield of pure monoflavianate was 0·5818 g. In both these experiments the arginine content, calculated from the product obtained on 1 recrystallization, was in excess of that known to be present, whereas if the recrystallization be continued until the operational loss is no more than 3·0 mg. and the appropriate correction be applied, the recovery is satisfactory. These results are not strictly in keeping with Vickery's deductions, and it became of interest to apply a similar treatment to products obtained directly from protein hydrolysates. In the following experiments Vickery's directions were adhered to as closely as possible and the flavianate was separated by using 5 mol. flavianic acid/mol. arginine present.

Arginine content of edestin. Details of four experiments, using different concentrations of protein, are given in Table 13. In the 1st the arginine content of the protein, calculated from the weight of recrystallized flavianate, was 16·75%, a value in excellent agreement with that of Vickery [1940] and also with that obtained in our comprehensive analysis (Table 3). The product, nevertheless, was not pure arginine monoflavianate, and in each of the four replicate determinations made it was necessary to recrystallize 5 times in all before the operational loss in weight was reduced to 4 mg. Allowing for overall losses (5 × 4 = 20 mg.) the corrected arginine content of the protein was 16·23 ± 0·03%. The other three experiments, in which different concentrations of protein were used, gave corrected values in close agreement with this figure.

This result was somewhat disconcerting, for our own experiments with a solution of arginine, confirming those of Vickery, show that the product obtained after only 1 recrystallization does not contain any arginine diflavianate. Appropriate Tristram-Block analysis of the collected edestin mother liquors from the 2nd to the 5th recrystallization moreover failed to disclose the presence of any appreciable amount of histidine. The extraneous material was clearly some other amino-acid flavianate, and indirect evidence in support of this is furnished by the fact that in these edestin experiments, and also in those of other proteins quoted later, the 1st recrystallization product was slightly hygroscopic (as Vickery records). According to our experience pure arginine monoflavianate, after drying in the oven at 102° and cooling to room temperature in a desiccator, gains no more than 1 mg./0·5 g. when exposed for 3 days to the atmosphere of the laboratory. In a recent paper, Vickery, Smith, Hubbell & Nolan [1941] cite experiments with water-melon globulin in which low results (17·44% as against 17·90%) were obtained when the original hydrolysate-flavianic acid mixture, after standing at 6–7° for 4 days, was allowed to stand at room temperature for a short time before filtration. They attributed this to resolution of arginine diflavianate, but from our experience we would suggest that it was the contaminants mentioned above that had been redissolved and that the lower value of 17·44% is the more reliable. In the experiments quoted in Table 13 the hydrolysate-flavianic acid mixture was allowed to stand for 4 days at +2° before filtration; a slightly higher temperature (6–7°) did not significantly affect the results.

Arginine content of egg albumin. The value based on the once recrystallized product is in close agreement with that obtained by Vickery [1940] if allowance is made for the N contents of the proteins concerned. The corrected value, based on the 4 times recrystallized product, is in agreement with that obtained during our comprehensive analysis (see Table 12). For this protein the differences concerned are small.

Arginine contents of horse and cattle haemoglobin. Clarification with charcoal of these two protein hydrolysates did not remove all the haem, which separated at once on adding flavianic acid to the aliquot (25 ml.) used for analysis. The precipitate was centrifuged off immediately and well washed. The collected centrifugate and washings (45 ml.), on standing, gave a voluminous precipitate of yellow crystalline material containing large amounts of histidine and lysine flavianates. After filtering off, the product was liberally washed with the usual wash solution, but in spite of this the recrystallized material was still heavily contaminated and 4 additional recrystallizations were needed to give pure arginine monoflavianate. The corrected value (Table 13) for the arginine content of horse carboxyhaemoglobin is a little higher than the 3·59 ± 0·07% recorded by Vickery [1940], while that for cattle haemoglobin is much higher than the 3·1% (on the globin) recorded by Bergmann & Niemann [1937].

Arginine content of amandin. Accepting 18·75% as the total N content of this protein [Chibnall *et al.*, 1943] our corrected value for arginine is 12·54 ± 0·05%. The value for arginine-N is 21·63% T.P.N.; Vickery [1940] records 22·1% T.P.N.

All these findings lead us to the conclusion that the Vickery procedure, if modified as we suggest, will give a high yield of arginine monoflavianate of unquestionable

Table 13. *Results of arginine analyses by Vickery's method*

Protein used	Edestin				Egg albumin	Horse carboxy-haemoglobin	Cattle haemoglobin
Wt. of protein in aliquot of hydrolysate (g.)	2·935	0·8288	0·97	0·97	2·665	3·565	3·76
Vol. used for precipitation (ml.)	50	50	25	50	25	45	45
Conc. of protein (g./100 ml.)	5·86	1·66	3·88	1·94	10·66	7·92	8·35
No. of determinations	4	2	4	2	4	2	3
Vol. used for recrystallization of flavianate (ml.)	50	50	25	50	25	50	50
Wt. of recrystallized flavianate (g.)*	1·379	0·381	0·5	0·445	0·4264	{0·4593 {0·4895	0·4516
Arginine content of protein (%)	16·75	16·4	16·5	16·34	5·71	—	—
No. of additional recrystallizations of flavianate	4	2	3	2	3	4	3
Final wt. of flavianate (g.)†	1·316	0·366	0·431	0·428	0·4105	0·3571	0·3659
Overall correction (g.)	0·02	0·012	0·012	0·012	0·012	0·02	0·016
Corrected arginine content of protein (%)	16·23	16·31	16·31	16·20	5·65	3·75	3·62

* In each analysis (except those of the haemoglobins) the replicates did not differ by more than 10 mg.
† In each analysis the replicates did not differ by more than 1 mg.

purity, yet we are not convinced that in all cases, especially when the hydrolysate contains a large amount of arginine, the separation is strictly quantitative. In other words, the method may not indicate with absolute certainty the number of arginyl residues in the protein molecule.

DISCUSSION

It will be seen from Tables 3, 4, 7 and 8, and from the text, that the working losses in the procedure we have adopted for the separation and estimation of the dicarboxylic acids and bases in protein hydrolysates have been reduced to what are probably the lowest possible limits. In the analysis of β-lactoglobulin, which required about 900 working hr. to take to completion, the gross overall loss of N amounted to only 1·25% T.P.N., a remarkable achievement considering the number of operations concerned, for it is less than that which most previous workers have incurred during the preliminary removal of the mineral acid used for hydrolysis. With edestin and egg albumin the corresponding stepwise losses were no greater, but the total overall loss was enhanced by the application of the Cu salt procedure for the removal of certain of the monoamino-acids and proline. Later experience suggests that this loss was greater than it need have been, for we used an unnecessarily large amount of $CuCO_3$ to make the Cu salts with the result that the relatively small amount of N retained by the bulky residue was not readily characterized. At the time these operations were performed the almost complete insolubility of the Cu salt of methionine in boiling water was not realized. In any future analysis we would suggest that the Cu salts at stage 14 be made by the cautious addition of $CuCO_3$ to the boiling solution under conditions that would permit of rapid filtration of the excess before the separation of this very insoluble salt of methionine, which could thus be obtained free from extraneous material. If the protein contains a polysaccharide moiety, as in egg albumin, or polyuronide impurities as in our forage crop preparations, reduction of Cu^{++} to give Cu_2O will occur almost immediately, but it should be possible to remove this with the slight excess of $CuCO_3$ by filtration.

The complete procedure is admittedly very laborious, yet we feel that our final data (Table 14) for edestin, egg albumin and β-lactoglobulin will be of value to those interested in protein analysis, for the errors involved are known within small limits and the results can thus be used to assess the accuracy of new or existing methods of estimation which are less onerous to perform. We have, indeed, already attempted this in our investigations on the dicarboxylic acids [Bailey *et al.*, 1943] and the bases (Section 5).

Table 14. *Dicarboxylic and basic amino-acid analysis of edestin, egg albumin and β-lactoglobulin*

(Small residual solubility corrections have been applied.)

	Edestin		Egg albumin		β-Lactoglobulin	
	N as % total protein-N	Wt. as % of protein	N as % total protein-N	Wt. as % of protein	N as % total protein-N	Wt. as % of protein
Glutamic acid	10·54	20·70	9·70	16·10	13·14	21·51
Aspartic acid	6·77	12·0	5·43	8·13	6·68	9·88
Arginine	28·85	16·71	11·44	5·63	5·95	2·89
Histidine	3·50	2·41	2·50	1·45	2·69	1·54
Lysine	2·44	2·37	6·15	5·06	12·07	9·75

SUMMARY

1. A method for the estimation of the dicarboxylic and basic amino-acids in protein hydrolysates is described, in which these amino-acids are isolated as pure derivatives and in quantitative yield by step-wise removal without overall loss.

2. The cardinal point in the procedure is that no reagent may be introduced into the main hydrolysate unless it can be quantitatively removed at a later stage without appreciable loss of N. Cu^{+} and Cu^{++} may be introduced into the hydrolysate and subsequently removed as Cu_2S and CuS respectively at any stage in the analysis; $SO_4^{=}$ must not be removed as $BaSO_4$, or Cl^{-} as AgCl until the hydrolysate has been cleared of cystine and the main part of the dicarboxylic acids.

3. In the analytical procedure adopted the cystine is first removed as the cuprous mercaptide of cysteine. The major part of the dicarboxylic acids is then isolated by a modification of Foreman's lime-ethanol method. Following this the bases are removed by precipitation with phosphotungstic acid and then estimated by the gravimetric procedure of Tristram-Block. The solubility factors inherent in the Foreman treatment for the dicarboxylic acid and in the phosphotungstate precipitation of the bases are overcome by repeating these operations at a later stage when the major part of the monoamino-acids and proline has been removed by various means from the collected mother liquors.

4. The method has been applied in turn, with modification suggested by experience, to hydrolysates of edestin, egg albumin and β-lactoglobulin; the overall losses of N were 2·95, 2·86 and 1·25% of the total protein-N respectively. The final values for glutamic acid, aspartic acid, arginine, histidine and lysine are given in Table 14.

5. The accuracy of the Tristram-Block procedure for the direct estimation of the bases and of Vickery's flavianic acid method for the direct estimation of arginine are criticized in light of experience.

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The Tryptophanase-tryptophan Reaction

6. CARBOHYDRATE-AMINO ACID RELATIONSHIPS CONCERNED IN THE INHIBITION OF INDOLE PRODUCTION BY GLUCOSE IN CULTURES OF *ESCHERICHIA COLI*

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The main facts which have been established previously by Happold & Hoyle [1936], and Evans, Handley & Happold [1941; 1942] are that *Escherichia coli* (*Bact. coli*), grown in a complex medium containing glucose and tryptophan, does not contain the tryptophanase enzyme system and that in a simple medium either phenylalanine or tyrosine is necessary to complete the glucose inhibition of the production of the tryptophanase system. A suggestion was made by Evans *et al.* [1942] that the inhibition of indole-production by cells grown in the presence of glucose and phenylalanine is maintained by the metabolism of stored carbohydrate. In this investigation we have attempted to substantiate this suggestion and also to determine the activity of the stereo-isomers of phenylalanine and tyrosine.

EXPERIMENTAL

The bacterial cultures. The Jebbs strain of *Escherichia coli* was used. The medium used for all but one of the investigations had the following composition: *l*-cystine, 1 mg.; glycine, 5 mg.; *dl*-valine, 10 mg.; *dl*-Na glutamate, 50 mg.; Na lactate, 150 mg.; NaCl, 50 mg.; $Na_2HPO_4.12H_2O$, 25 mg.; KH_2PO_4, 3·5 mg.; $MgSO_4.7H_2O$, 3 mg.; $FeSO_4.7H_2O$, 4 μg.; distilled water to 10 ml.; *p*H adjusted to 7·6. Glucose and tryptophan were added as sterile solutions to give a concentration of 1 and 0·003 % respectively, whilst phenylalanine, where used, was in a concentration of 0·01 %. The total volume of medium used in any particular experiment was 100 ml. Cultures were made in Roux bottles.

Estimation of polysaccharide. A washed suspension of the cells was prepared and its volume noted. A portion of this suspension was taken to obtain the total dry weight of organisms. The polysaccharide content of the remainder was determined by the method of Sahyun [1931]. The following are representative results for the estimation of known amounts of glycogen by this method in our hands:

Glycogen present mg.	Glycogen estimated mg.
8·5	7·4
4·4	3·5
2·2	1·6
1·0	1·1
0·4	0·4

These results indicated that the method was sensitive for small amounts of glycogen of the order of 1 mg. but for larger amounts the degree of recovery was only about 80 % and larger amounts of norite had to be used. Since the amount of glycogen estimated in the following experiments was of the order of 1 mg., the method was sensitive enough for our purposes.

Influence of phenylalanine on the metabolism of Esch. coli

(*a*) *Effect of added phenylalanine on oxygen uptake.* Evans *et al.* [1941], using the Van Slyke method for the estimation of amino-N, reported that phenylalanine was not metabolized by *Esch. coli*. This has now been examined with the Barcroft respirometer. 0·5 ml. of a washed suspension of *Esch. coli* grown on casein agar was added to 0·5 ml. of a 0·5 % solution of phenylalanine and 4·0 ml. K phosphate buffer (*p*H 7·6) contained in a Barcroft cup. The following results were obtained:

	μl./hr.			
O_2 uptake of cells with phenylalanine substrate	82	86	87	100
O_2 uptake of cells with no substrate	64		63	29

This difference in O_2 uptake, we suggest, corresponds either with the metabolism of phenylalanine or to the acceleration of the metabolism of some substance stored in the cell brought about by the phenylalanine.

(*b*) *Effect of phenylalanine on metabolism of glucose.* With phenylalanine and phenylalanine-free media, the glucose utilization of *Esch. coli* during a period of 24 hr. growth gave the following results:

Phenylalanine-free medium		*dl*-Phenylalanine (0·01 %) medium	
Total glucose utilization (mg.)	Glucose utilization (mg./mg. dry bact. wt.)	Total glucose utilization (mg.)	Glucose utilization (mg./mg. dry bact. wt.)
327	3·3	288	4·3
474	7·4	426	7·6
211	4·3	176	4·2

The total glucose metabolized was greater in the absence of phenylalanine and in general total growth was also greater, though this was less marked.

(*c*) *Effect of phenylalanine on polysaccharide storage.* The *dl* and the *l* forms of phenylalanine were used. The results obtained were as follows:

Cells grown in					
Phenylalanine-free medium		*dl*-Phenylalanine (0·01 %) medium		*l*-Phenylalanine (0·01 %) medium	
Polysacc. content (mg./mg. dry bact. wt.)	Total dry bact. wt. (mg.)	Polysacc. content (mg./mg. dry bact. wt.)	Total dry bact. wt. (mg.)	Polysacc. content (mg./mg. dry bact. wt.)	Total dry bact. wt. (mg.)
0·03	19	0·045	20	0·02	27
0·025	92	0·04	112	0·027	118
0·02	53	0·03	31	0·02	27
0·02	48	—	—	0·02	46
0·03	44	0·05	42	—	—
0·01	53	0·03	55	—	—

Thus *dl*-phenylalanine increased the polysaccharide storage of *Esch. coli*, but the naturally occurring *l*-form was inactive in this respect.

(*d*) *Effect of phenylalanine on the metabolism of stored polysaccharide.* Whilst the previous results indicated that there was a difference in the polysaccharide content of the two types of cells, they gave no indication that this polysaccharide was metabolizable. Cells were therefore grown on phenylalanine-free and phenylalanine-containing media, and their O_2 usage estimated in the absence of external substrate by means of a Barcroft respirometer. Typical results were as follows:

Oxygen uptake of cells grown on	
Phenylalanine-free medium μl./hr./mg. dry bact. wt.	*dl*-Phenylalanine medium μl./hr./mg. dry bact. wt.
16·7	41·7
11·0	42·3
29·0	78·0
22·5	36·0
22·5	44·0

A greater amount of some metabolizable substance is present in cells grown in the phenylalanine-glucose medium than in those grown in the phenylalanine-free medium. To confirm that the metabolism of stored polysaccharide did take place during the lag period before indole was produced, when cells grown in a glucose-phenylalanine medium were incubated with tryptophan, the following experiment was performed:

Washed cell suspensions of *Esch. coli* grown in phenylalanine-free and phenylalanine-containing media were prepared. Part of each of these suspensions was incubated with tryptophan and the oxygen consumption of the remainder was determined. The cells grown in the phenylalanine-free medium rapidly produced indole whilst the other cells had a lag period. The O_2 consumption of each of these two types of cells for this lag period was determined. The following are the results obtained:

Oxygen uptake of cells grown on	
Phenylalanine medium μl./hr./mg. dry bact. wt.	Phenylalanine-free medium μl./hr./mg. dry bact. wt.
36·0	18·8
44·0	18·8
40·0	20·0

The average difference between the O_2 uptakes of these two types of cell is 20·8 μl./hr./mg. dry bacterial wt. The amount of glycogen which would require this amount of O_2 for its complete combustion would be approx. 0·025 mg./mg. dry bacterial wt. This amount of glycogen is of the same order as the difference between the polysaccharide content of the cells grown in the two types of media. Therefore we can state that in all probability the increase in polysaccharide content of *Esch. coli* under the influence of phenylalanine corresponds with an increase in the metabolizable polysaccharide stored, and that this polysaccharide is metabolized before the development of a tryptophanase system can take place in the cell.

Influence of tryptophan and tyrosine

(*a*) *Effect of tryptophan on the polysaccharide content.* In this series of experiments the phenylalanine concentration was kept constant at 0·01 %, and the tryptophan content was varied. The following are the results obtained:

Indole production	Tryptophan present in medium %	Polysaccharide content (in duplicate) mg./mg. dry bact. wt.	
–	0·000	0·03	
–	0·003	0·035	0·03
Trace	0·01	0·03	0·025
+	0·02	0·01	0·01

Tryptophan exerts an action antagonistic to that of phenylalanine in stimulating polysaccharide storage in the organism, and evidently the stoichiometric relationship which exists between these two amino-acids in the production of indole in cultures containing glucose is due to this antagonism.

(*b*) *Effect of tyrosine on the polysaccharide content.* The same type of experiment as already described

for phenylalanine was repeated using *dl*- and *l*-tyrosine. The following results were obtained:

Cells grown in					
Tyrosine-free medium		*dl*-Tyrosine medium		*l*-Tyrosine medium	
Polysacc. content (mg./dry bact. wt.)	Total bact. wt. (mg.)	Polysacc. content (mg./dry bact. wt.)	Total bact. wt. (mg.)	Polysacc. content (mg./dry bact. wt.)	Total bact. wt. (mg.)
0·010	40	0·027	54	0·016	74
0·029	39	0·055	26	—	—
0·025	31	0·071	25	0·042	15

Thus results are similar to those obtained with phenylalanine but are less marked.

Influence of mannose

(*a*) *Effect of mannose on the polysaccharide content.* A comparison between the ability of mannose and glucose to allow storage of carbohydrate in *Esch. coli* has been made by determining the effect of replacing glucose in the medium by mannose. Indole was consistently produced in the mannose medium and not in the glucose medium when *dl*-phenylalanine was present. The following are the results obtained:

Polysaccharide content (mg./mg. dry bact. wt.) of cells grown in			
dl-Phenylalanine present		*dl*-Phenylalanine absent	
Glucose medium	Mannose medium	Glucose medium	Mannose medium
0·027	0·013	0·014	0·007
0·034	0·010	0·013	0·010

The range of bacterial dry wt. was from 32 to 82 mg., with the major part falling between 40 and 50 mg. *dl*-Phenylalanine appeared to be unable to increase polysaccharide storage with mannose to any marked extent. There was no evidence of storage with mannose alone.

(*b*) *Metabolism of mannose by* Esch. coli. Ashford & Holmes [1929] showed that in brain there were two mechanisms for glycolysis, one concerned with the breakdown of glycogen and requiring the presence of inorganic phosphate, whilst the other was concerned with the breakdown of glucose and did not require the presence of inorganic phosphate. Subsequently Ashford [1933] showed that whilst the anaerobic breakdown of glycogen and glucose could take place at the same time without any signs of mutual inhibition, mannose and glucose under similar conditions could not be metabolized simultaneously. Thus the conclusion was drawn that the same enzyme system was concerned in the metabolism of both glucose and mannose. In such circumstances both sugars were presumably metabolized by the non-phosphorylating route. In the previous experiments we have shown that glucose in the presence of *dl*-phenylalanine is able to increase the polysaccharide storage in *Esch. coli* and we wondered whether, in this case, the glucose was being metabolized by the phosphorylating route. On the other hand, since mannose is unable to effect such storage it might be metabolized by the non-phosphorylating route. To test this the oxygen consumption of washed suspensions of *Esch. coli* grown on phenylalanine-free glucose medium was determined, using glucose, mannose and a combination of the two as substrates. The concentration of the sugars was 0·5 %. To a similar series phenylalanine was added to give a concentration of 0·01 %. The results obtained were as follows:

Oxygen uptake of cells in the presence of					
Phenylalanine and			No phenylalanine		
Glucose Q_{O_2}	Mannose Q_{O_2}	Glucose + mannose Q_{O_2}	Glucose Q_{O_2}	Mannose Q_{O_2}	Glucose + mannose Q_{O_2}
11·3	12·1	12·5	12·1	9·8	11·7
7·3	7·6	11·4	11·0	9·0	10·0
10·3	12·3	12·4	14·3	14·5	13·3
5·6	5·9	5·9	—	—	—

Q_{O_2} = μl. oxygen/hr./mg. dry bact. wt.

No apparent summation of O_2 consumption takes place when the cells are acting upon the mixed substrate. Mannose is slightly more readily metabolized than glucose in the presence of phenylalanine and the reverse is true in its absence. The O_2 consumption of the cells metabolizing glucose is apparently greater when phenylalanine is absent from the medium. This conforms with its action in enhancing polysaccharide storage.

DISCUSSION

From the results presented there would appear to be no doubt that the mechanism of the glucose inhibition of the tryptophanase system in *Esch. coli* depends on the ability of the cell to store more polysaccharide than is present in the cell after culture in indole-producing media and that the metabolism of such stored polysaccharide may delay the onset of indole production when such cells are incubated with tryptophan.

Antagonistic actions on the storage of polysaccharide appear to be exerted by tryptophan and phenylalanine, the latter stimulating storage. Thus the suggestion can be made that a labile component of the enzyme systems concerned in the breakdown of tryptophan to indole and in the storage of carbohydrate is common to both and that competition occurs. The role which *dl*-phenylalanine plays in stabilizing the enzyme system so that storage of carbohydrate becomes its main function is obscure.

The 'natural' *l*(−) form appears to be inactive and therefore it is presumably the 'non-natural' *d*(+) form which exerts this action.

Butts, Dunn & Hallman [1938], working with rats, found that *dl*-phenylalanine was very active in the formation of liver glycogen in this animal, although *dl*-tyrosine was found to be inactive. Subsequently Butts, Sinnhuber & Dunn [1941] found that although *dl*-tyrosine was inactive in the formation of liver glycogen, the *l*(−) form caused some storage to occur. Our experiments may indicate that *l*(−)-tyrosine is more active in enhancing polysaccharide storage in *Esch. coli* than is *l*(−)-phenylalanine but in neither case does the *l*(−) form show an activity equal to the activity shown by the *dl* form. The similarities and dissimilarities of the action of phenylalanine and tyrosine on the carbohydrate metabolism of a mammal and of a bacterium open up some interesting questions.

dl-Phenylalanine inhibits to a slight extent the dissimilation of glucose by washed suspension of *Esch. coli*. If the amount of polysaccharide storage depends on a balance between the rate of storage and the rate of dissimilation, then if the latter rate is decreased we shall have increased storage. If we accept the scheme postulated by Hanes [1940] for the metabolism of glycogen by yeast cells, and the generally accepted scheme for the dissimilation of glucose, and apply them to the current problem, then phenylalanine may act either by influencing the equilibrium of the reaction glycogen⇌glucose-1-phosphate, or the dissimilation of the latter. Increase in glycogen storage may be sufficient to explain the decrease in the dissimilation of glucose, or the decrease in the dissimilation of glucose phosphate may be sufficient to explain the increased glycogen.

With regard to the failure of mannose to produce polysaccharide storage in *Esch. coli* under the influence of *dl*-phenylalanine, it would appear true to say that mannose is apparently metabolized by the same route as glucose, and since in this case glucose is capable of forming polysaccharide, it would appear most likely that this route is a phosphorylating one. It should, however, be pointed out that Cori, Schmidt & Cori [1939] prepared an eluate of yeast which together with the addition of adenylic acid and glucose-1-phosphate could produce a polysaccharide. Such a mixture could not, however, produce a polysaccharide from a similar mannose phosphate ester. Thus the conclusion to which we come is that although the mannose may be undergoing metabolism by the phosphorylating route, the enzyme system concerned in polysaccharide formation is unable to form polysaccharide from the mannose-1-phosphate which may be formed.

SUMMARY

1. *dl*-Phenylalanine and *dl*-tyrosine bring about an increased storage of metabolizable polysaccharide in *Esch. coli* when the latter is grown on a medium of known composition containing tryptophan and glucose.

2. The *l*(−) forms of these amino-acids are inactive in bringing about this increased polysaccharide storage.

3. Mannose is unable to increase the polysaccharide storage of *Esch. coli* in the presence of *dl*-phenylalanine, and suggestions have been given to explain this.

4. Tryptophan is antagonistic to *dl*-phenylalanine in its action on polysaccharide storage.

5. Suggestions have been put forward to localize the site of activity of *dl*-phenylalanine, and also to relate the tryptophanase system and the systems concerned in carbohydrate breakdown.

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The Effect of the Plasma Proteins upon the Sedimentation Rate of Human Blood

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Fresh blood which contains an anticoagulant will separate out on standing, leaving a sharp boundary between the clear plasma above and the packed red cells below. The rate of this sedimentation is used as a clinical test, because in the healthy subject it occurs very slowly, while in many pathological conditions it is extremely rapid. From the work of Fahraeus [1929], Tiffeneau & Gysin [1937] and Frazer & Rennie [1941] it is known that the rate of sedimentation is determined by the protein constituents of the plasma, but no clear correlation has yet been found between the observed sedimentation rate and the quantities of the various protein fractions [Alred-Brown & Monroe, 1935; Ropes, Rossmeisl & Bauer, 1939; Frazer & Rennie, 1941]. In this paper the problem has been tackled by building up 'pathological plasmas' with protein fractions isolated from normal plasma and observing the behaviour of normal cells suspended in them.

METHODS

Estimation of sedimentation rate. The following procedure gave consistent results. Blood was collected into a tube containing dry anticoagulant in such amount that 100 ml. of blood contained 0·3–1·0 g. of Na citrate, or 0·5–3·0 g. of heparin. Within 15 min. of withdrawing the blood it was suspended in a Westergren type of tube, giving a column 200 mm. high and 2·8–3·0 mm. in diameter. This was fixed vertically in a rack, and the sedimentation rate expressed in terms of the height in mm. of plasma visible above the erythrocytes at a given time.

Preparation of protein fractions. The material used was normal human plasma which had been citrated and kept for about 6 days at 4°. The salted-out precipitates were dialysed under pressure against 0·85% NaCl until no contaminant salt remained, and the proteins sterilized by Seitz filtration and stored at 4°. Fibrinogen, total globulin, euglobulin, pseudoglobulin and total albumin were precipitated by appropriate concentrations of $(NH_4)_2SO_4$; crystalbumin, globoglycoid and seromucoid were separated as described by Hewitt [1938*a*], and by Rimington & van den Ende [1940], and nucleo-protein by the CO_2 precipitation method of Plimmer [1926]. All these fractions gave Molisch and cystine-S reactions in accordance with the literature. Seroglycoid could not be obtained, and if it does exist in human plasma it will be included in our total albumin fraction, and will not be present as a contaminant in any subsidiary fraction. Hewitt's seroglycoid may be equivalent to our seromucoid.

Our experiments were always duplicated, and usually repeated three or more times on different occasions, using separate batches of the protein in question, and different normal cells. The variation in the results was usually less than ±9%.

RESULTS

Influence of 'purified' plasma proteins on rate of cell sedimentation

(*a*) *Influence of mode of preparation of total globulin fraction on its action on sedimentation.* Fahraeus [1918], and later Tiffeneau & Gysin [1937], showed that if cells were suspended in solutions of protein fractions isolated from plasma, the sedimentation rates obtained were in the order, fibrinogen > globulin > albumin. Before attempting to repeat these results, experiments were carried out to ascertain whether the treatment undergone by a protein fraction during isolation affected its sedimentation-influencing properties. The results obtained with the total globulin fractions separated from portions of the same batch of normal plasma by three different methods (Table 1) showed substantial differences, despite the fact that the pseudoglobulin/euglobulin ratios were the same (4/1) for all three preparations.

Table 1. *Rate of sedimentation of human erythrocytes in the presence of serum globulin prepared by different methods*

(Total globulin conc. = 3 g./100 ml. 0·85% NaCl. Cell vol. = 20%.)

	Total globulin fractions prepared by precipitation by		
	Sat. with NaCl	Sat. with $MgSO_4$	½-sat. with $(NH_4)_2SO_4$
Sedimentation after 30 min. (mm.)	10	15	5

(*b*) *Action of single plasma proteins prepared as described under Methods.* We repeated the experiments of Fahraeus [1918] and of Tiffeneau & Gysin [1937], using a greater range of plasma protein fractions. From the results shown in Table 2, it will be seen that the most rapid sedimentation rates are obtained with fibrinogen and euglobulin. The question arose whether the 'slower' proteins, such as total albumin, when mixed with a 'fast' protein, merely reduced the sedimentation rate by acting as a diluent, or if the resulting sedimentation rate was the mean of those for the two separate proteins.

Table 2. *Rate of sedimentation of human erythrocytes suspended in 'pure' solutions of normal plasma protein fractions*

(Protein conc. = 3 g./100 ml. 0·85 % NaCl. Cell vol. = 20 %.)

Protein	Average sedimentation (mm.) after 1 hr.	4 hr.	24 hr.	No. of exp.
Fibrinogen	100	150	150	3
Total globulin:				
(*a*) Pseudoglobulin/euglobulin ratio = 4:1	4	22	100	2
(*b*) Pseudoglobulin/euglobulin ratio = 1·6:1	14	20	130	2
Euglobulin	20	50	70	3
Pseudoglobulin	2	16	50	4
Total albumin	1·5	8	26	3
Crystalbumin	2	10	40	2
Globoglycoid	2	5	26	1
Seromucoid	3	7	37	1
Nucleoprotein	3	8	40	2

(*c*) *Interaction of two plasma proteins.* For experiments with fibrinogen three tubes were set up containing (*a*) fibrinogen (3 g./100 ml.); (*b*) the same fibrinogen solution diluted with an equal volume of saline, and (*c*) the fibrinogen solution diluted with an equal volume of a 3 g./100 ml. solution of total albumin. The results (Table 3) show that albumin

Table 3. *Rate of sedimentation of human erythrocytes suspended in solutions of fibrinogen diluted with saline or albumin*

(Fibrinogen and albumin sol. = 3 g./100 ml. 0·85 % NaCl. Cell vol. = 20 %. Results mean of 5 experiments.)

	Equal vol. of Fibrinogen and fibrinogen	Fibrinogen and 0·85 % NaCl	Fibrinogen and albumin
Sedimentation after 1 hr. (mm.)	100	40	6·0

does not act simply as a diluent; if the sedimentation rate in the mixture were the mean between that in fibrinogen (100 mm.) and that in albumin (1·5 mm.), the reading in the third tube should have been 50·7 mm. Actually the albumin inhibits the action of the fibrinogen so strongly that the reading is scarcely more than that given by a 3 % solution of albumin alone. The action of other proteins upon fibrinogen was therefore studied, in order to ascertain whether this inhibitory action was peculiar to albumin.

The series of experiments in Table 4 shows that globulin slightly accelerates the sedimentation rate in fibrinogen solutions, and that this effect is due to the euglobulin fraction. The inhibiting effect of the albumin is caused by globoglycoid, while crystalbumin and seromucoid have hardly more effect than a mere saline dilution. The effect of nucleoprotein

Table 4. *Sedimentation of human erythrocytes suspended in solutions of fibrinogen mixed with equal volumes of another protein fraction*

(Protein conc. = 3 g./100 ml. 0·85 % NaCl.)

Protein mixture	Mean sedimentation at end of 1st hr. mm.	No. of exp.
Fibrinogen and saline	40	2
Fibrinogen and total globulin (pseudoglobulin/euglobulin ratio = 4:1)	60	2
Fibrinogen and euglobulin	75	2
Fibrinogen and pseudoglobulin	30	3
Fibrinogen and total albumin	4·6	4
Fibrinogen and crystalbumin	50	3
Fibrinogen and globoglycoid	10	2
Fibrinogen and seromucoid*	70	1
Fibrinogen and nucleoprotein	1·0	3

* Seromucoid 1·8 %.

is surprising, for after 24 hr. the fall is only 3 mm. Its normal concentration is low (0·003 g./100 ml. plasma), and since it is insoluble at 20 % sat. $(NH_4)_2SO_4$, it will occur as a contaminant in the fibrinogen fraction.

These results suggest that the interaction of proteins in the plasma has a great influence upon erythrocyte stability. The effect of combining any two protein fractions in equal proportions has been worked out in some detail, and the corresponding saline dilutions given for comparison. The results tabulated in Table 5 are expressed as the fall at the end of 3 hr. with a 20 % volume of fresh normal cells. The 3rd hr. reading is presented here because the 'slower' proteins fail to show significant differences at 1 hr., and the 'faster' have all reached the terminal packing stage by the 5th hr. To find the sedimentation for, say, a combination of fibrinogen and euglobulin, take the horizontal fibrinogen row of figures and follow it along until it intersects the vertical column for euglobulin. The reading in this square is 142 mm., which represents the average 3rd hr. fall for this combination. Table 5 shows the action of the proteins upon fibrinogen and also the inhibitory effect of total albumin upon the globulins, which is not so great in proportion as with fibrinogen. Nucleoprotein gives a greater sedimentation rate alone than when mixed with fibrinogen. We can give no explanation of this, but the experiment was repeated three times with the same result. The interaction between the 'slower' proteins is uninteresting. The figures show the effect of two proteins only in solution: in plasma, however, all the fractions are coexistent, and as we have shown that albumin inhibits fibrinogen, it seems likely that albumin will also inhibit the euglobulin which, if alone, would accelerate the fibrinogen, and thus exercise a double inhibition upon fibrinogen.

Table 5. *Sedimentation of human erythrocytes suspended in mixtures containing equal volumes of two plasma proteins*

(Protein conc. = 3 g./100 ml. 0·85% NaCl. Results given as mean of 2–4 experiments.)

	Mean sedimentation after 3 hr. mm.									
	Nucleo-protein	Sero-mucoid	Globo-glycoid	Cryst-albumin	Total albumin	Pseudo-globulin	Euglo-bulin	Total globulin	Fib-rinogen	Saline
Saline	—	—	3·5	—	5	7	10	7	97	3
Fibrinogen	3	130	40	100	10	60	142	130	150	97
Total globulin	—	—	6	7	7	9	20	11	130	7
Euglobulin	—	—	5	7	2	10	25	20	142	10
Pseudoglobulin	—	—	3	35	5	15	10	9	60	10
Total albumin	—	—	3	3	5	5	2	7	10	5
Crystalbumin	—	—	2	6	3	35	7	7	100	—
Globoglycoid	—	—	3	2	3	3	5	6	40	3·5
Seromucoid	—	5	—	—	—	—	—	—	130	—
Nucleoprotein	5	—	—	—	—	—	—	—	3	—

(*d*) *Interaction of three or more plasma proteins.* This type of interaction was worked out in three stages:

(1) Fibrinogen altered: Constant globulin and albumin.

(2) Globulin and its fractions altered: Constant fibrinogen and albumin.

(3) Albumin and its fractions altered: Constant fibrinogen and globulin.

The effect of fibrinogen upon sedimentation rate was studied by comparing the fall of cells in plasma with that in serum from the same person, part of the specimen being collected with oxalate and part being allowed to clot for serum. The oxalated cells were centrifuged, washed in saline, and resuspended in the plasma and the serum. Table 6 shows that

Table 6. *Comparison of sedimentation rates of human erythrocytes suspended in plasma and in serum from the same blood*

(Five experiments performed.)

	Erythrocytes suspended in	
	Plasma	Serum
Normal blood, cell vol. reduced to 20%. Sedimentation at 7th hr. (mm.)	15	6
Pathological blood, cell vol. unaltered. Sedimentation after 1 hr. (mm.)	60	6

although some sedimentation can take place in the absence of fibrinogen, it plays the chief part, and therefore its acceleration or inhibition will have a great effect upon sedimentation. Oakley [1938] was able to add normal blood of known fibrinogen content to blood which completely lacked fibrinogen, and found that the relation between increased sedimentation rate and fibrinogen content was practically linear.

The second and third types of reaction were studied by constructing artificial plasma from a mixture of single protein fractions. In order to test the accuracy of this method, sedimentation rates on normal and pathological blood were carried out parallel with analyses on the plasma, and records of cell size and cell volume kept. From these data artificial plasma was built up, the correct volume of fresh normal cells suspended in it, and the sedimentation rate determined. The total protein percentages were often lower than in the original, because it was found difficult to obtain all the individual fractions sufficiently concentrated, but the ratio of the component fractions was maintained. The protein solutions must be in good condition, for a denatured batch of, say, fibrinogen will invalidate the whole experiment. The results of imitating normal and pathological blood are shown in Tables 7 and 8.

Table 7. *Sedimentation of human erythrocytes suspended in natural or artificial normal plasma*

	Composition of natural normal plasma proteins as g./100 ml.	Composition of artificial normal plasma g./100 ml.
Fibrinogen	0·3	0·24
Globulin	2·7	2·16
Albumin	4·0	3·2
Total protein	7·0	5·6
Cell vol. (%)	40·0	40·0
Cell diam. (μ)	7·1	7·1
Sedimentation after 1 hr. (mm.)	4·0	3·0

From these two experiments we conclude that an artificial mixture of normal proteins does behave similarly to natural pathological plasma, in its effect upon sedimentation.

The globulin fractions were studied on the basis of the tuberculous plasma analysis given in Table 8. It will be seen that the fibrinogen and albumin were maintained constant and total globulin first replaced by euglobulin, and then by pseudoglobulin.

Table 8. *Sedimentation of human erythrocytes suspended in natural and artificial tuberculous plasma*

	Composition of plasma (proteins as g./100 ml.)			
		Artificial plasma containing		
	Natural plasma	Total globulin	Eu-globulin	Pseudo-globulin
Fibrinogen	0·6	0·6	0·6	0·6
Total globulin	3·32	3·2*	—	—
Euglobulin	2·15	—	3·2	0
Pseudoglobulin	1·17	—	0	3·2
Albumin	3·6	3·6	3·6	3·6
Total protein	7·52	7·4	7·4	7·4
Cell vol. (%)	40·0	40·0	40·0	40·0
Sedimentation after 1 hr. (mm.)	94·0	90·0	80·0	26·0

* Prepared by precipitation by $MgSO_4$.

Because euglobulin slightly accelerates the sedimentation rate of fibrinogen (Table 4) we consider that the artificial plasma with euglobulin ought to have shown a greater sedimentation rate than total globulin, and attribute the slightly lower reading to a poor batch of the protein. The inhibitory action of the pseudoglobulin is, however, clear enough. Some months after this experiment was performed, a case of tuberculosis became available in which the sedimentation rate was low and the pseudoglobulin high, while the disease was sufficiently advanced for the sputum to show great numbers of tubercle bacilli. The results were as follows:

	Plasma protein content	
Protein	Conc. g./100 ml.	
Fibrinogen	0·5	
Euglobulin	0·1	Cell vol. = 36 %
Pseudoglobulin	2·3	Sedimentation (1 hr.) = 0·5 mm.
Albumin	4·49	

This analysis may well be compared with that for plasma and the artificial plasma containing pseudoglobulin (Table 8).

A typical nephritic blood was constructed in order to study the effect of the component fractions of albumin. This was chosen because there is much known about the albumin changes in nephritis and nephrosis. The fibrinogen and globulin were kept constant throughout; normal total albumin was used first to see if a typically rapid nephritic sedimentation could be obtained.

The sedimentation (Table 9) is not so great as one would expect in natural blood with the same composition. The total albumin was then replaced by globoglycoid and then by crystalbumin. The plasma containing globoglycoid (Table 9) gave an extraordinarily slow sedimentation, and the crystalbumin mixture caused a far more rapid fall than could be obtained in real nephritic blood. The interesting point is that the component fractions of albumin have a remarkably different effect upon the sedimentation rate. Unfortunately, seromucoid could not be prepared in sufficiently concentrated solution for use in these experiments.

Table 9. *Sedimentation of human erythrocytes suspended in artificial nephritic plasma*

	Composition of artificial plasma (proteins as g./100 ml.) containing		
	Total albumin	Globo-glycoid	Cryst-albumin
Fibrinogen	0·32	0·26	0·32
Globulin	3·2	2·6	3·3
Total albumin	1·7	0·0	0·0
Crystalbumin	0·0	0·0	1·6
Globoglycoid	0·0	1·3	0
Total protein	5·2	4·2	4·2
Cell vol. (%)	20·0	20·0	20·0
Sedimentation after 1 hr. (mm.)	15·0	1·0	145·0

Hewitt [1938*a*] analysed nephritic serum and found the crystalbumin fraction to be far below normal, while the globulin and seroglycoid (equivalent to seromucoid?) were not strikingly different; no mention, however, is made of globoglycoid. If crystalbumin is reduced in real nephritic blood, one would expect, from these experiments, a slower sedimentation rate. Unfortunately, there is no convenient method of analysing the albumin components on a small sample of blood, and our results do not tally with his findings.

DISCUSSION

From this series of experiments we conclude that the power of sedimentation of blood is not controlled by the absolute concentration of either the total plasma proteins or the protein fractions, but by *the inhibition of one protein by another*. Thus it is quite possible to have a high fibrinogen content and low sedimentation rate due to increase of pseudoglobulin at the expense of the euglobulin, while the total globulin concentration remains stationary; or an increase of globoglycoid over crystalbumin can inhibit the action of the globulins without inducing any abnormality in the gross protein fractions. This concept probably explains why Alred-Brown & Monroe [1935] and Ropes *et al.* [1939] found no relation between the concentration of any one protein fraction and sedimentation rate, while Frazer & Rennie [1941] found decrease of albumin and, to a lesser extent, increase of fibrinogen, associated with rise of sedimentation rate, while the globulins had no appreciable effect.

Our experiments suggest that when the blood sedimentation rate is raised, there is no alteration in the chemical structure of the proteins. A possible exception to this is the Bence-Jones protein [Devine, 1941]. Geottsch [1936] found that nephritic total globulin was immunologically different from the normal, and Alving [1936] found changes in the cystine content of total albumin in nephrosis. Hewitt [1938*b*] later showed that in both cases the individual fractions of globulin or albumin give normal chemical and immunological reactions, and the altered ratios are responsible for the abnormality of the plasma.

SUMMARY

1. The proteins from normal plasma were separated into nine fractions, and the rate of fall of a 20% vol. of erythrocytes in 3% solutions of the proteins during 1 hr. was found to range from 100 mm. for fibrinogen to 1·5 mm. for total albumin.

2. The fractions were combined in equal amounts using 39 pairs. The action of the 'faster' fractions, fibrinogen and euglobulin, is inhibited by the 'slow' nucleoprotein and globoglycoid.

3. Artificial pathological plasmas built up from these fractions gave sedimentation rates similar to those for the comparable natural material. With artificial tuberculous plasma it was found that by altering only the component globulin fractions, the sedimentation rate could be changed from 94 to 26 mm.; and with artificial nephritic blood, substitution of total albumin by crystalbumin altered the rate from 15·0 to 145 mm., while substitution of the total albumin by globoglycoid changed it from 15·0 to 1·0 mm.

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Phosphorolysis and Synthesis of Glycogen in Animal Tissues

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The mechanism of glycogenolysis in muscle and liver is described by Cori, Cori & Schmidt [1939] as follows:

(1) Glycogen + phosphate ⇌ glucose-1-phosphate (catalysed by phosphorylase and adenylic acid).

(2) Glucose-1-phosphate ⇌ hexose-6-phosphate (catalysed by phosphoglucomutase and Mg^{++}).

(3) Hexose-6-phosphate ⇌ glucose + phosphate (catalysed by phosphatase in liver).

(3*a*) Hexose-6-phosphate ⇌ hexose-1:6-diphosphate → lactic acid (in muscle).

These reactions are responsible for glucose formation in liver, and are the first steps in glycolysis in muscle. Glycogen resynthesis too is probably explained by the same reactions [Cori & Cori, 1941]. It seemed of interest, therefore, to study the distribution of the enzymes concerned, in various tissues at various stages of development, and to compare the enzymic activities with the rate of metabolism of glycogen in the tissue concerned. The further question was also investigated in this connexion whether insufficiency of one or more of the designated enzymes is the cause of metabolic disturbances in certain pathological conditions.

A separate series of experiments dealt with some properties of phosphorylase, and in particular with its inhibition by glucose. This inhibition was first observed by Lehmann [1938] who suggested its possible physiological importance. It may be responsible for the homeostatic mechanism described by Soskin, Essex, Herrick & Mann [1938].

METHODS

Preparation of phosphorylase

Phosphorylase was prepared according to Cori *et al.* [1939] by adsorbing the enzyme of tissue extracts on $Al(OH)_3$ and eluting with Na glycerophosphate. It was found, however, that C_γ $Al(OH)_3$ prepared according to Willstätter, Kraut & Erbacher [1925] frequently yielded inactive enzyme preparations. With the C_β variation, on the other hand, active preparations were regularly obtained, and remained active for long periods provided the precipitate had been washed only once with NH_4OH instead of twice.

Estimation of enzymic activity

(*a*) *Phosphorylase activity* was estimated on the basis of the amount of glycogen synthesized from glucose-1-phosphate in the presence of the enzyme. A mixture of 1 vol. enzyme solution and ½ vol. of a solution containing 3% glucose-1-phosphate and 1½% glycogen in *N*/10 H_2SO_4 is incubated at 25° for various periods. In some cases 0·25 mg. adenylic acid is added per ml. of the mixture. The extent of glycogen synthesis was determined by measuring the amount of inorganic phosphate liberated.

(*b*) *Phosphoglucomutase activity* was estimated as the decrease in easily hydrolysable phosphate (10 min. hydrolysis with *N*/1 HCl at 100°) when glucose-1-phosphate is added to 0·3 g. of tissue brei suspended in 2 ml. of bicarbonate Ringer containing *M*/40 NaF, and incubated at 37° with continuous shaking. The bicarbonate Ringer is composed of 4 ml. 1·15% KCl, 1 ml. 0·8% $MgSO_4$, 100 ml. 0·9% NaCl and 60 ml. 1·3% $NaHCO_3$. Before use the solution is saturated with CO_2.

(*c*) *Glycogen phosphorolysis.* The conversion of glycogen into hexose-6-phosphate was measured by estimating the disappearance of inorganic phosphate when 0·3 g. of tissue brei was incubated with 1% glycogen and *M*/40 NaF in phosphate-Ringer at 37° with continuous shaking. The phosphate-Ringer is similar in composition to the bicarbonate-Ringer except that phosphate buffer *M*/10 of *p*H 7·4 is used instead of bicarbonate.

(*d*) *Estimation of phosphate.* Phosphate was estimated according to Lohmann & Jendrassik [1926] after the reactions had been stopped by the addition of 1 ml. 20% $CCl_3.COOH$. In the case of glycogen synthesis from glucose-1-phosphate the acid solution is immediately diluted to avoid splitting of the ester by acid.

RESULTS

Distribution of the phosphorolytic system

Phosphorylase was found in almost all tissues (Fig. 1). Organs with a highly active glycogen metabolism, such as muscle, liver and heart, however, give much more active phosphorylase preparations than organs which are devoid of, or have only a slight glycogen metabolism, as, for example, lungs, skin, testes, intestine, spleen, adipose tissue and placenta. Kidney and brain occupy an intermediary position as regards both phosphorylase activity and glycogen metabolism.

The distribution of phosphoglucomutase and glycogen phosphorylase is generally parallel to the

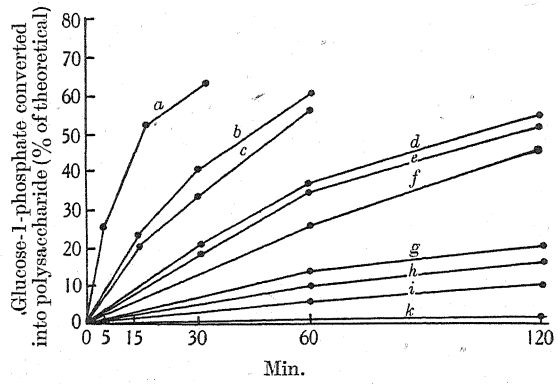

Fig. 1. Glycogen synthesis from glucose-1-phosphate by phosphorylase in tissue brei. *a*, muscle; *b*, heart and subcutis; *c*, liver; *d*, kidney; *e*, brain; *f*, tumour; *g*, lung and intestine; *h*, skin, testes and spleen; *i*, adipose; *k*, placenta.

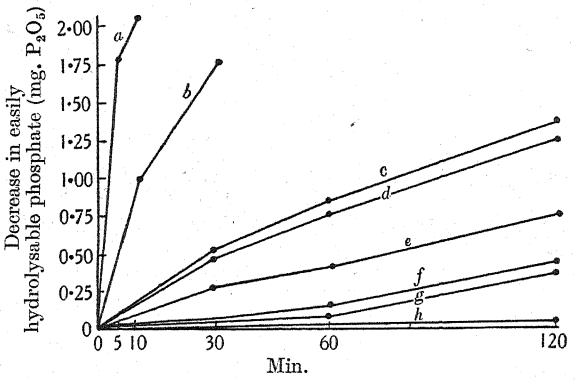

Fig. 2. Conversion of glucose-1-phosphate into hexose-6-phosphate in tissue brei. *a*, muscle; *b*, heart; *c*, skin; *d*, brain; *e*, spleen; *f*, lung; *g*, tumour; *h*, intestine, adipose and testes.

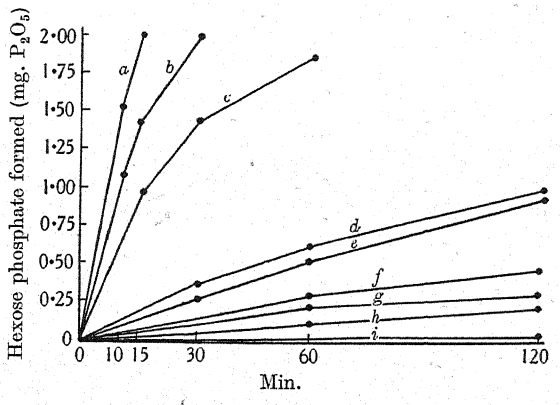

Fig. 3. Phosphorolysis of glycogen in tissue brei. *a*, muscle; *b*, liver; *c*, heart; *d*, brain; *e*, kidney; *f*, skin; *g*, lung; *h*, tumour; *i*, intestine, testes, adipose, pancreas and placenta.

intensity of glycogen metabolism. Yet several exceptions were observed (Fig. 2, 3). Tumour tissue (sarcoma), though it is not generally classed as a glycogen-metabolizing tissue, displayed a high phosphorylase activity. The phosphorolytic breakdown of glycogen by this tissue is, however, very slow as compared with that of other tissues. This apparent discrepancy is due to the low phosphoglucomutase activity of tumour tissue.

Glycogen metabolism of subcutaneous tissue

A further apparent disagreement occurs with subcutaneous tissue. This tissue is one of the most active as regards the phosphorylase activity, but was not previously known to possess any high glycogen-metabolizing capacity. It was found, however, that this tissue has a regular and high glycogen metabolism (Table 1), and that subcutaneous tissue from normal rats contains quantities

Table 1. *Glycogen content of subcutaneous tissues*

Treatment of animal	No. of exp.	Blood sugar (mg./100 ml.)	Average glycogen content of subcutaneous tissues %
Normal rats	24	121	0·27
1–2 days' fast	12	99	0·06
0·03 mg. adrenaline 1–2 hr. previously	5	175	0·06
0·005–0·01 mg. adrenaline 1–2 hr. previously	5	125	0·05
6 ml. 25% glucose *per os*, in addition to regular diet	8	110	0·50

of glycogen comparable with those of muscle tissue. This glycogen store is readily mobilized during fasting and thus becomes depleted. On administration of adrenalin the glycogen content drops. This decrease was observed with doses of adrenalin causing an elevation of blood sugar, as well as with low doses having no marked effect on the blood-sugar level. Sugar administration by stomach tube caused a marked elevation of the glycogen content of the subcutaneous tissues.

When rats received a normal, carbohydrate-rich diet after prolonged undernutrition (causing 20% loss of body wt.), an exceptionally high glycogen content was found in the subcutaneous tissues on the 1st day after recommencement of the normal feeding. After 7 days on the usual carbohydrate-rich diet the glycogen content is again normal. In Table 2 the results with subcutaneous tissue are compared with those for glycogen in adipose tissue and in liver, under similar conditions [Tuerkischer & Wertheimer, 1942].

Subcutaneous tissue may thus be regarded as a glycogen-metabolizing tissue, and appears to constitute a supplementary glycogen store for the body.

The development of the phosphorolytic system

It is known, largely from the work of Needham and his collaborators [Needham & Lehmann, 1937; Needham & Nowinski, 1937], that embryonic tissue acts preferably on glucose, and only very slowly on glycogen, to form lactic acid. Chick embryo is rarely able to phosphorylate glycogen. The glycogenolytic system in various organs develops only with advancing age. Experiments were undertaken in order to identify the rate-limiting component of the glycogenolytic system, and to clarify the course of its development.

In muscle tissue from rats up to 10 days of age no phosphorylase activity could be detected (Table 3). This lack of activity is not due to the lack of the coenzyme adenylic acid, since even after addition of optimal amounts of coenzyme only an insignificant glycogen synthesis took place. As Table 3 shows, muscle phosphorylase activity rises rapidly after the 10th day and is substantial by the 14th day. At this stage the amount of adenylic acid present in muscle is the limiting factor, since the addition of this substance caused an acceleration of glycogen synthesis which is never observed in adult muscle tissue.

Table 3. *Glycogen synthesis in muscle tissue from young rats*

	Glucose-1-phosphate converted into glycogen in 1 hr. (% theoretical)	
Age in days	Without addition of adenylic acid %	With addition of adenylic acid %
10	0	5
14	26	45
20	25	52

Table 2. *Glycogen content of subcutaneous tissues after normal feeding of rats previously subjected to prolonged undernutrition*

	Glycogen content of tissue (%)							
	After 7 days' undernutrition wt. loss = 20%	Days after re-feeding with carbohydrate-rich diet						
Tissue		1	2	3	4	5	6	7
Subcutaneous tissue	0·07	0·58	0·92	0·61	0·51	0·50	0·55	0·32
'White' adipose tissue	0·00	0·55	0·90	0·50	0·23	Trace	0·00	0·00
'Brown' adipose tissue	0·00	1·85	2·40	1·80	0·27	Trace	0·00	0·00
Liver	0·40	7·2	6·3	6·0	3·5	3·1	—	—

The rate of development of the glycogenolytic system in brain is quite different from that in muscle. The activity of the various components of the phosphorolytic system in relation to the activity of normal adult tissue—taking the last as 100—is shown in Fig. 4. It can be seen that in contrast to

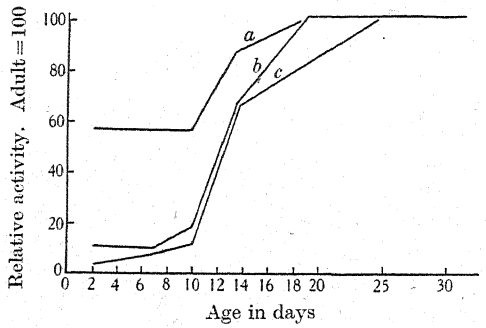

Fig. 4. Phosphorolysis, glycogen synthesis, and phosphoglucomutase action in brain of rats at different ages. *a*, glycogen synthesis; *b*, phosphorolysis; *c*, phosphoglucomutase.

the results with muscle, the glycogen-synthesizing capacity is already present immediately after birth to the extent of about 50% of the adult value. Nevertheless, no phosphorolytic breakdown of glycogen takes place at this age. This is accounted for by the complete lack of phosphoglucomutase activity. Thus, the glucomutase and not the phosphorylase is the limiting factor in glycogenolysis in the brain of young rats. As Fig. 4 shows, the phosphorolytic capacity of brain parallels the development of the mutase activity. Following an initial period of a very slow development up to the age of 10 days, the mutase begins to develop quickly, and at 14 days about 70% of the adult activity is reached. After this age the results become less uniform. In general, the adult value for both mutase and glycogen phosphorolysis is reached at an age between 20 and 30 days. The phosphorylase system develops independently. Its relative activity was always higher than other systems examined. Hence, it can at no stage be regarded as the limiting factor of glycogen metabolism during development.

It remained to be established whether the development of the enzymic activities is due to the development of new kinds of cells or is the result of formation of new enzymes in cell types already present at birth. For this purpose the activity of white and of grey brain matter was compared. The results (Table 4) show that no significant difference in glycogen phosphorolysis can be observed in white and grey matter of brain. The development of the phosphorylase system thus seems to take place in all parts of the brain and not be specially due to the development of the grey matter.

Table 4. *Phosphorolysis of glycogen in white and grey matter of rabbit brain*

Tissue	Hexose phosphate formed (mg. P_2O_5) in 30 min.	60 min.	120 min.
(1) White matter	0·50	0·85	1·00
Grey matter	0·48	0·65	0·95
(2) White matter	0·60	0·85	0·98
Grey matter	0·64	0·93	1·10
Nerve fibre	0·48	0·63	0·70

Phosphorolysis in pathological conditions

Since it had been shown that a parallelism exists between the capacity of an organ to metabolize glycogen and the activity of its phosphorolytic system, it was of interest to determine whether a deficiency in the glycogen phosphorolytic system could be found in pathological cases in which glycogen metabolism is disturbed.

The effect of liver damage produced by various methods was first examined.

Table 5. *Phosphorolysis of glycogen in animals subjected to liver damage*

Animal	Experimental treatment	Initial glycogen content %	Fat content %	Hexose phosphate formed in brei (mg. P_2O_5) during 15 min.	30 min.	60 min.
(*a*) Liver tissue:						
Guinea-pig	None	2·4	4	1·32	2·05	2·45
,,	CCl_4	0·4	8	0·70	1·45	1·60
,,	,,	0·3	9	0·70	1·43	1·63
,,	,,	0·0	8	0·48	0·87	1·23
,,	,,	0·0	9	0·42	0·78	1·10
,,	P	2·0	5	0·95	1·50	2·25
,,	,,	Trace	8	0·62	1·24	1·82
,,	,,	,,	11	0·48	0·55	0·86
,,	,,	,,	10	0·54	0·68	0·94
Rabbit	None	2·0	4	1·10	1·82	2·32
,,	CCl_4	0	7	0·30	0·77	0·93
,,	,,	0	9	0·00	0·00	0·40
,,	$CHCl_3$	0·4	9	0·25	0·53	0·85
,,	,,	0·5	8	0·37	0·64	0·98
,,	Phlorrhizin (fasting)	0·0	8	0·82	1·36	1·67
,,	,,	0·2	9	0·75	1·24	1·75
(*b*) Muscle tissue:						
Guinea-pig	None	—	—	1·45	2·03	2·40
,,	CCl_4	—	—	1·67	2·50	2·55
,,	,,	—	—	1·33	2·05	2·18
,,	,,	0	8	1·45	2·10	2·40
,,	,,	0	9	1·30	2·08	2·43
,,	P	2·0	5	1·80	2·60	2·80
,,	,,	Traces	8	2·44	2·74	2·94
,,	,,	,,	11	1·68	2·30	2·56
,,	,,	,,	10	1·63	2·00	2·40

It is evident from the data (Table 5) that livers damaged by poisons such as CCl_4, P and $CHCl_3$ show impaired glycogen phosphorolysis breakdown. The fat and glycogen contents of liver served as measures of liver damage. It may be seen that when the liver fat has been raised above 8%, with low glycogen content, by the administration of the above poisons, the phosphorolytic capacity of the liver is far below normal. When fat accumulation was induced in the liver without damage, by prolonged fasting and phlorrhizin treatment, no comparable loss of phosphorolytic capacity was observed.

The diminution in phosphorolytic activity does not seem to be due to inactivation of phosphorylase, since, even in cases of almost complete lack of phosphorolytic activity, phosphorylase preparations from the same liver synthesize glycogen normally. The diminution may be due to an inactivation of the phosphoglucomutase. Further proof of this assumption is not available, since the estimation of phosphoglucomutase in liver is not practicable, liver phosphatase being active even in the presence of high concentrations of fluoride. In liver brei glucose-1-phosphate is split to give inorganic phosphate without the accumulation of hexose-6-phosphate.

Phosphorolysis in muscle tissue from adrenalectomized and thyroidectomized rats

Phosphorolysis was examined in muscles of adrenalectomized rats maintained in good condition by Rubin-Krieck's salt solution. Rats were used 10–30 days after adrenalectomy, and estimations were carried out after 10–30 min. incubation of the brei. Phosphorolysis in muscle of such rats was either normal or slightly diminished. The results agree with those of Holmes & Lehmann [1940] who, working on liver, found no loss of phosphorolysis activity in minced liver of adrenalectomized rabbits. They contradict those of Verzar & Montigel [1942], who found that phosphorolysis in the muscles of adrenalectomized rats was almost completely abolished when short incubation times (up to 30 min.) were used. It has, however, been shown by Holmes & Lehmann [1940] that the glycogen synthesis from glucose by rabbit-liver slices is lowered when the animals used were adrenalectomized. Bendall & Lehmann [1942] obtained the same result with rat-liver slices; they could restore the normal activity by a previous injection with extract from the suprarenal cortex.

Thyroidectomized rats also showed normal phosphorolytic power. The rats were maintained before and after operation on a diet containing fresh milk. Similar results were obtained throughout an interval of 1–4 months after operation.

In thyrotoxicosis also, phosphorolytic activity was found to be normal.

Properties of the phosphorylase system

(*a*) *Heat inactivation.* The phosphorylase of muscle of rats and rabbits is highly thermolabile. Heating to 60° for 5 min. causes complete destruction of the enzyme. At 55°, 20 min. were needed for complete destruction, whereas at 50° even after 60 min. some activity was preserved.

(*b*) *Ultra-violet irradiation.* Phosphorylase from rabbit muscle was irradiated by a mercury lamp at a distance of 50 cm. for 30 min. The temperature was not allowed to rise above 30°. Controls were maintained under the same conditions but covered to prevent penetration of ultra-violet light. The irradiation caused partial or complete inactivation of phosphorylase activity.

(*c*) *Inhibition by glucose.* In confirmation of the results of Lehmann [1938], Gill & Lehmann [1939], Lehmann [1939] and of Cori & Cori [1940], glycogen phosphorylation was found to be inhibited by glucose. A concentration of glucose of about 2% caused almost complete inhibition. On the other hand, in contradiction to Cori's results, glucose inhibition was found to be counteracted by adenylic acid, as is shown in Tables 6 and 7.

Table 6. *Inhibition by glucose of phosphorolysis in incubated muscle brei; influence of adenylic acid in counteracting this inhibitory action of glucose*

Material added	Hexose phosphate formed in 15 min. mg. P_2O_5	Observed inhibition of hexose phosphate formation (% of max. possible)
None	2·13	0
Glucose (initial conc. = 2%)	0·35	84
Adenylic acid (3 mg./ml.)	1·97	0
Adenylic acid (3 mg./ml.) plus glucose (initial conc. = 2%)	1·35	30

Na lactate, as well as Na pyruvate, caused only partial inhibition of glycogen synthesis even at concentrations as high as 2%. Adenosine-5-phosphoric acid (referred to here as adenylic acid) cannot be replaced by adenosine-3-phosphoric acid as a counteractant of glucose inhibition.

The discrepancy between these results and those of Cori is explained by the fact that Cori used lower concentrations of glucose. As is shown in Table 7, when concentrations of 0·3% glucose are used no influence of adenylic acid is detected. Addition of adenylic acid causes the reactivation of the enzyme only to a certain degree, so that after addition of adenylic acid the same activity is found with 2% as with 0·3% of glucose.

The inhibition of phosphorylase by glucose cannot be explained simply by competition between glucose

Table 7. *Inhibition by glucose of glycogen formation in muscle eluate; influence of adenylic acid in counteracting this inhibitory action of glucose*

Incubation period (min.)	Material added: Glucose (initial conc. %)	Material added: Adenylic acid (mg. added to 1 ml. eluate)	Formation of glycogen from added glucose-1-phosphate (% of theoretical)	Inhibition of glycogen formation from glucose-1-phosphate (% of max. possible)
10	0·0	0·0	44	0
	2·0	0·0	1	98
15	0·0	0·0	46	0
	2·0	0·0	7	85
	2·0	1·5	30	35
	2·0	(1·5*)	8	83
	0·0	0·0 plus Na lactate (initial conc. = 2%)	34	26
	0·0	0·0 plus Na pyruvate (initial conc. = 2%)	24	48
20	0·0	0·0	53	0
	2·0	0·0	3	95
20	0	0	25	0
	0·3	0	15	40
	2·0	0	2	92
	0	1·5	53	0
	0·3	1·5	35	34
	2·0	1·5	35	34

* Adenosine-3-phosphate.

and glucose-1-phosphate for the enzyme. Further proof for this is given by experiments with potato phosphorylase. This phosphorylase, like other phosphorylases from plants, is not activated by adenylic acid. Competition between glucose and glucose-1-phosphate should exist in this system as well as in animal phosphorylases, and phosphorylase action should be inhibited. If, however, glucose inhibition is connected with activation by adenylic acid, no inhibition by glucose should be observed with potato enzyme. The results in Table 8 show that the latter alternative applies.

Table 8. *Inhibition by glucose of glycogen formation in eluate of potato juice*

Incubation period (min.)	Glucose added (initial conc. %)	Formation of glycogen from added glucose-1-phosphate (% of theoretical)	Inhibition of glycogen formation from glucose-1-phosphate (% of max. possible)
15	0·0	43	0
	2·0	41	5
30	0·0	58	0
	2·0	62	0

(*d*) *Nature of polysaccharide synthesized by phosphorylase.* The polysaccharide synthesized by animal phosphorylase is generally glycogen, that synthesized by plant phosphorylase starch. This has been deduced by Cori *et al.* [1939] and by Hanes [1940] from the colour given with iodine, and has since been confirmed by X-ray spectrum analysis [Baer & Cori, 1941]. An exception to this rule is constituted by muscle phosphorylase, which acts on glucose-1-phosphate with formation of starch. We have been able to show that, prior to the formation of starch by muscle phosphorylase, a polysaccharide is formed which gives the yellow-pine colour with iodine typical of glycogen. This reaction cannot be demonstrated with fresh phosphorylase preparations acting at room temperature because starch formation in this case is too quick. If, however, the enzyme preparation is left overnight in the ice-box before it is allowed to act on its substrate, or if incubation is carried out at 0°, the yellow-pine colour reaction can be demonstrated after 5–10 min. of incubation. The colour becomes increasingly masked by the blue iodine reaction as synthesis proceeds further.

DISCUSSION

Good agreement has been found between the intensity of glycogen metabolism and the phosphorylase and phosphoglucomutase activity of various tissues. This finding favours the assumption that glycogen metabolism in all animal tissues follows the path described for muscle and liver by Cori *et al.* [1939]. An unexpectedly high phosphorylase activity and intense glycogen metabolism is shown by subcutaneous tissue.

In rat muscle almost no phosphorylase activity could be demonstrated immediately after birth. Muscle phosphorylase activity becomes considerable at the age of 14 days, when the muscles also begin to function normally. In brain, on the other hand, phosphorylase activity is already practically maximal at birth. Nevertheless, glycogen phosphorolysis in brain brei at this stage is very slow. The apparent discrepancy was found to be attributable to the lack of active phosphoglucomutase. When the mutase activity develops to normal, 14–20 days after birth, glycogen phosphorolysis too was found to become normal. This development of glycogen phosphorylation activity parallels the rise in O_2 consumption [Fazekas, Alexander & Himwich, 1941], and increases as the phosphatatic and glycolytic activities decline [Cohn & Kaplan, 1941; Epelbaum & Skvirskaya, 1940] with advancing age.

In poisoning with P, CCl_4 or $CHCl_3$, the glycogen metabolism of the liver is disturbed. In severe cases no glycogen at all could be found in liver. This is correlated with a marked fall in phosphorolytic capacity, apparently due to a decrease in the activity of phosphoglucomutase. The phosphorylase activity of liver in such cases is almost normal.

SUMMARY

1. A modified method for the preparation of phosphorylase by adsorption on $Al(OH)_3$ is described. By this method, phosphorylase can be demonstrated in all animal tissues.

2. The phosphorylase activity and the intensity of the glycogen metabolism of a tissue are shown to be positively correlated. The same is true also of the phosphorolytic breakdown of glycogen.

3. Subcutaneous tissue possesses a highly active phosphorylase, and metabolizes glycogen at a high rate.

4. Phosphorylase is not found in the muscles of rats less than 10 days of age. In 14-day-old rats the enzyme is already comparatively active.

5. Brain tissue from new-born rats shows 60 % of the phosphorylase activity of the adult brain. Phosphoglucomutase in new-born rat brain, however, was either inactive or absent. This enzyme is the limiting factor in glycogen phosphorolysis up to 14–20 days after birth, after which time its activity approaches that found in the adult brain.

6. In intoxication by phosphorus, carbon tetrachloride and chloroform, liver phosphoglucomutase is inactivated, but phosphorylase activity is almost normal.

7. In adrenalectomized as well as in thyroidectomized rats no decrease in glycogen phosphorolysis by muscle could be demonstrated.

8. Some properties of the phosphorylase of muscle have been studied: on irradiation with ultra-violet light, or on heating to 55° for 20 min., complete destruction of the enzyme occurred. Adenylic acid diminished the inhibition caused by glucose in 2 % concentration. With 0·3 % glucose no lessening of inhibition was found when adenylic acid was added. With potato phosphorylase no inhibition of glycogen synthesis by 2 % glucose was found.

9. If synthesis by muscle phosphorylase is slowed down by low temperature or by ageing of the enzyme preparation, an iodine colour reaction such as is given by glycogen can be observed after 5–10 min. of synthesis.

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The Chemical Behaviour of Dehydro-*l*-ascorbic Acid *in vitro* and *in vivo*

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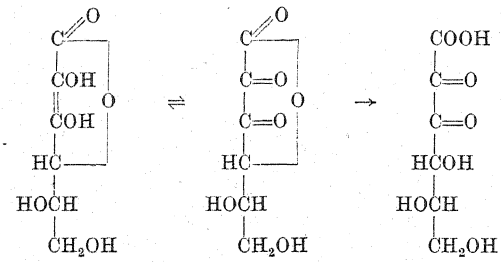
(*Received 29 March 1943*)

It is reasonable to assume that the function of *l*-ascorbic acid in the metabolism of the animal organism is that of a link in a co-ordinated chain of reactions. It might therefore be expected that, since ascorbic acid and dehydro-*l*-ascorbic acid constitute under certain conditions a thermodynamically reversible oxidation-reduction system, dehydroascorbic acid would become a component of these co-ordinated reactions, although under prevailing conditions it may be present in too small quantities to be detected by the available technique. With this hypothesis in view one of us started some years ago an investigation on the behaviour of dehydroascorbic acid in the guinea-pig. It was then found that whilst ascorbic acid could be almost completely recovered from the organs and tissues

of the guinea-pig soon after injection, this was not the case when dehydroascorbic acid was used. It was therefore assumed that when dehydroascorbic acid (II) was present in the organism in large quantities a part of it was reduced in the body to ascorbic acid (I) but considerable quantities were at the same time converted irreversibly into 2:3-diketo-*l*-gulonic acid (III).



I *l*-Ascorbic acid — II Dehydro-*l*-ascorbic acid — III 2:3-Diketo-*l*-gulonic acid

Before this could be demonstrated experimentally it was necessary to acquire further information on the subject. First, a direct method for estimating diketogulonic acid in small quantities in animal tissues had to be devised. In the second place, the chemistry of the conversion of dehydroascorbic acid had to be studied in greater detail than hitherto in order to be able to interpret correctly results obtained *in vivo*. The former has since been accomplished [Penney & Zilva, 1943]. The latter investigation is reported here. This communication is divided into three sections, one dealing with the purely chemical aspect of the problem performed *in vitro*, the second with the behaviour of dehydroascorbic acid and diketogulonic acid *in vivo*, and the third consisting of a general discussion of the results described in the first two sections.

The conversion of dehydroascorbic acid to diketogulonic acid was first postulated by Herbert, Hirst, Percival, Reynolds & Smith [1933] who observed that dehydroascorbic acid mutarotated in acid solution. For convenience we therefore often employ the expression mutarotation for the change of dehydroascorbic acid into diketogulonic acid, although the conversion was not measured by us polarimetrically.

I. EXPERIMENTS *IN VITRO*

Since this investigation pivots on the colorimetric determination of diketogulonic acid [Penney & Zilva, 1943] it was essential to elucidate as far as possible the chemical nature of the reactions involved. This estimation consists of two distinct phases, namely, the formation of the 2:4-dinitrophenylhydrazine derivative and the subsequent production of a red coloration on addition of alkali. The colour forms the ultimate criterion for the assessment of diketogulonic acid.

Solutions of dehydroascorbic and diketogulonic acids were prepared as described [Penney & Zilva, 1943]. To 250 ml. solutions containing 100 mg. of each of these compounds and of ascorbic acid, three equivalents of 2:4-dinitrophenylhydrazine in 100 ml. 2 *N* HCl were added. After 5 days at 40° the bulky red precipitates were filtered off, washed with dilute HCl and water and dried *in vacuo* over H_2SO_4. Table 1 gives the crude yields of the preparations and their melting points after recrystallization from acetone-ethanol. The mixed M.P. was unchanged. These melting points agree well with those obtained by Herbert *et al.* [1933] for ascorbic and dehydroascorbic acid derivatives (282° and 280° decomp. respectively), and by Drumm, Scarborough & Stewart [1937] for the dehydroascorbic acid derivative (280°). Roe [1937], on the other hand, found that the dehydroascorbic acid derivative melted at 257–259° decomp. This worker apparently did not recrystallize his product, which might explain the lower M.P. The M.P. of the diketogulonic acid derivative obtained by us was the same as those of the ascorbic and dehydroascorbic acid derivatives.

Table 1. *Crude yields and* M.P. *of 2:4-dinitrophenylhydrazine derivatives*

Derivative from	Crude yield % of theory	M.P. corrected
Ascorbic acid	80	279° decomp.
Dehydroascorbic acid	85	280° ,,
Diketogulonic acid	70	281° ,,

The N content of the diketogulonic acid derivative (Weiler) was 20·8 % (calculated for osazone 21·0%), which is also in good agreement with those obtained by the above workers for the ascorbic and dehydroascorbic acid derivatives.

Small quantities of each of the derivatives in 50 % acetone-ethanol were passed through a column of active alumina (Savory & Moore). In each case the adsorption was similar, a purple zone being obtained at the top of the column which could not be eluted with acetone, but was easily removed with acetic acid. No chromatographic separation could be obtained from a mixture of the three derivatives.

In view of the above results, little doubt remains that the same dinitrophenylhydrazine derivative is obtained from each of the three compounds. Indirect evidence further points to the fact that in this reaction, ascorbic acid is first oxidized to dehydroascorbic acid which is converted to diketogulonic acid by the opening of the lactone ring. That ascorbic acid is first oxidized to dehydroascorbic acid is shown by the fact that more than two equivalents of the dinitrophenylhydrazine are required

to obtain the maximum yield. Furthermore, as will be shown later, at the *p*H of the reaction mixture the mutarotation of dehydroascorbic acid is accelerated. All this evidence suggests that the dinitrophenylhydrazine ultimately condenses with diketogulonic acid in the case of all the three compounds, in support of which we found that when the H^+ concentration of the reacting solution was increased, the rate of the reaction as measured by the colorimetric method also increased (Table 2) as a consequence of the more rapid formation of diketogulonic acid.

Table 2. *Colour produced by dehydroascorbic acid in the presence of 0·2 N and 0·04 N HCl at 38°, recorded as reciprocal colorimeter readings*

HCl conc.	Reciprocal colorimetric reading after				
N	60	90	120	180	270 min.
0·2	0·0145	0·024	0·0325	0·0435	0·068
0·04	0·010	0·017	0·025	0·0325	0·0545

In another experiment the disappearance of dehydroascorbic acid was determined in the total absence of, and in the presence of, an excess of dinitrophenylhydrazine under the same conditions, i.e. in 0·4 *N* HCl at 38°. The solutions were withdrawn after various periods, cooled, and a slight excess of furfuraldehyde added to remove the remaining dinitrophenylhydrazine. Precipitation of this furfuraldehyde derivative was complete in 5 min. $KHCO_3$ was then added to neutralize partially the acid present and the final *p*H adjusted to 3·5 by the addition of McIlvaine's phosphate-citrate buffer. After filtration, the dehydroascorbic acid was determined in both cases by treatment with H_2S and subsequent titration with indophenol. The presence of furfuraldehyde in these experiments did not interfere with the determination of dehydroascorbic acid. The results are given in Table 3; it

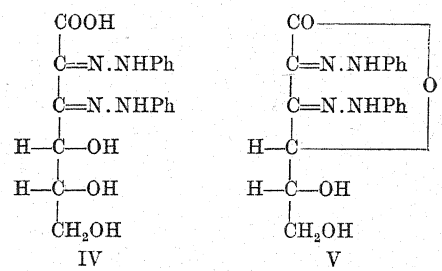
Table 3. *Recovery of dehydroascorbic acid after mutarotation in the presence and in the absence of 2:4-dinitrophenylhydrazine at 38°. HCl conc. = 0·4 N*

	Dehydroascorbic acid found (%) after					
	30	60	90	120	150	180 min.
Dinitrophenylhydrazine present	80	64	53	44	41	32
Dinitrophenylhydrazine absent	80	65	55	46	40	34

will be seen that the quantity of dehydroascorbic acid remaining in each case was the same within the limits of the experimental error, thus demonstrating that dinitrophenylhydrazine does not react with dehydroascorbic acid directly. The necessity for converting ascorbic acid and dehydroascorbic acid to diketogulonic acid before the reaction can take place accounts for the differential rate of the formation of the diketogulonic acid derivative, and therefore affords the possibility of determining quantitatively diketogulonic acid in the presence of the other two compounds under suitable conditions.

The evidence obtained concerning the mechanism of the formation of the deep red coloration on the addition of alkali to the dinitrophenylhydrazine derivative of diketogulonic acid was less instructive. Although the dinitrophenylhydrazine derivative of diketogulonic acid was soluble in NaOH, it was insoluble in $KHCO_3$ and Na_2CO_3. A compound or a mixture of compounds melting about 85° could be obtained from the NaOH solution by acidification. This product, unlike the original dinitrophenylhydrazine derivative, was soluble in Na_2CO_3 and $KHCO_3$ solutions. The solution of the precipitated compound turned from yellow to deep red at *p*H 9·5–10·5. It is of interest to note that the dinitrophenylhydrazone of pyruvic acid similarly changes its colour over this range, which suggests the presence of a carboxylic group in the compound.

The fact that the dinitrophenylhydrazine derivative of diketogulonic acid did not react with $KHCO_3$ or Na_2CO_3 suggests the absence of a COOH group, and from this it may be concluded that the derivative possesses either a lactone or pyrazolone structure. Ohle & Böckmann's work [1934] on the reaction of araboascorbic acid with phenylhydrazine favours the former view. They found that in the presence of HCl both the open chain (IV) and the lactone (V) forms are present and that on simple recrystallization the former readily lactonizes.



It is quite likely that under the experimental conditions prevailing in the colorimetric determination of diketogulonic acid the corresponding open-chain compound (IV) is formed, which tends to lactonize in time or on crystallization. It is therefore the open-chain compound which in all probability reacts with NaOH to form the characteristic red colour.

Mutarotation of dehydroascorbic acid under different conditions

Before investigating whether any conversion of dehydroascorbic acid takes place *in vivo* it was necessary to study in detail the mutarotation of

this substance under different conditions so as to be able to interpret the results of metabolic experiments.

Mutarotation in acid medium at 25°. It was obviously of importance to ascertain the influence of concentrations of dehydroascorbic acid on its rate of mutarotation since it could not be assumed that the kinetics of the conversion to diketogulonic acid conformed to that of a reaction of the first order. By following colorimetrically the rate of mutarotation of three concentrations of dehydroascorbic acid, i.e. 50, 200 and 1000 mg./100 ml., it was observed that the velocity of conversion (%) was increased with increase of the concentration (Fig. 1). Further experiments, however, showed that this increase was not due to the increase in concentration of the dehydroascorbic acid but to the increase in the H^+ concentration of the medium, since the dehydroascorbic acid used in these experiments was prepared by oxidation of ascorbic acid with I_2. This reaction produces HI, the concentration of which would rise as higher concentrations of ascorbic acid were employed. That the rate of mutarotation is actually accelerated by a rise in the H^+ concentration of the medium is seen from Fig. 2 which shows the effect of the addition of various concentrations of HCl. In this experiment, 15 ml. HCl, of a concentration necessary to give the final normalities recorded in Fig. 2, were introduced into each of a series of tubes kept in a water-bath at 25°. Dehydroascorbic acid solutions (100 ml.) were prepared by the oxidation of ascorbic acid (100 mg.) with I_2. The temperature of the dehydroascorbic acid solution was rapidly adjusted to 25° and 5 ml. then added to each tube. At given times, tubes were withdrawn, $2N$ $KHCO_3$ added to adjust the pH of the contents to 2 and the final volume made up to 50 ml. Diketogulonic acid was then determined directly by the colorimetric method, and indirectly by reduction of the residual dehydroascorbic acid with H_2S. When Cl_2 was used as the oxidizing agent instead of I_2, identical results were obtained.

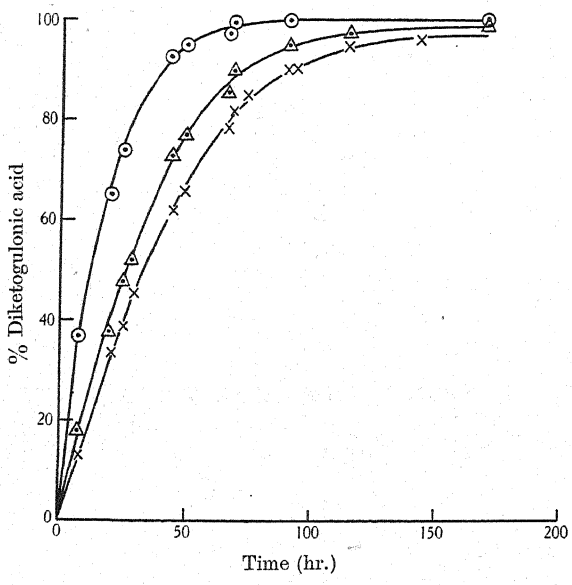


Fig. 1.

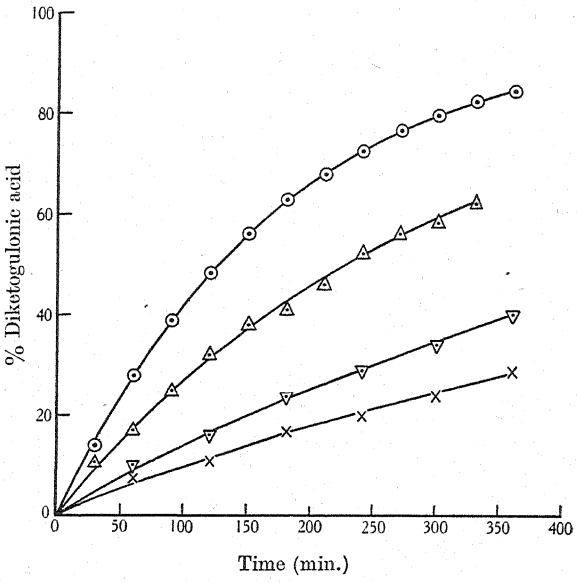


Fig. 2.

Fig. 1. Influence of initial concentration of dehydroascorbic acid on the rate of diketogulonic acid formation at 25° in the presence of HI produced during the oxidation of ascorbic acid with I_2. ×—× 50 mg./100 ml. △—△ 200 mg./100 ml. ⊙—⊙ 1 g./100 ml.

Fig. 2. Influence of hydrogen-ion concentration on the rate of mutarotation of dehydroascorbic acid at 25°. ⊙—⊙ In N HCl. △—△ In 0·5 N HCl. ▽—▽ In 0·2 N HCl. ×—× In 0·1 N HCl.

Further confirmation that the rate of conversion (%) is influenced by H^+ concentration was obtained in an experiment in which ascorbic acid was oxidized with I_2 or with KIO_3. In the former case the mutarotation was followed, with and without neutralizing the HI produced, whilst in the second case HCl equivalent to the HI produced by an equal quantity of ascorbic acid oxidized with I_2 was added to one of the neutral solutions of dehydroascorbic acid. It will be seen from Fig. 3 that the rate of conversion

(%) in each case was higher in acid solution than in neutral and was independent of the method of preparation.

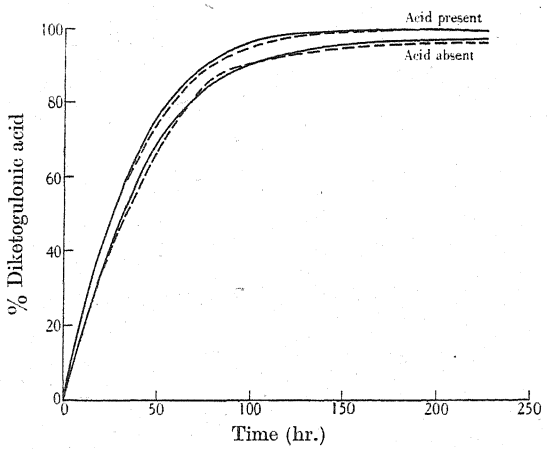


Fig. 3. —— Oxidizing agent I_2. ---- Oxidizing agent KIO_3.

The results in Table 4 show conclusively that the rate of conversion (%) of dehydroascorbic acid to diketogulonic acid is independent of concentration at a given H^+ concentration. This being so it would

Table 4. *Effect of initial concentration of dehydroascorbic acid on the mutarotation at 25°*

Initial conc. of dehydroascorbic acid mg./100 ml.	HCl conc. *N*	Diketogulonic acid (%) found after (hr.) 30	49	73
50	0·011	58	79	84
100		56	77	87
250		58	78	85
750		58	79	85
		2	3	4
25	1·0	48	63	72
100		50	66	74
200		50	65	76

be expected that the mutarotation should proceed as a reaction of the first order. By calculating all the results of this section according to the formula

$$k = \frac{2{\cdot}3 \log a/(a-x)}{t}$$

(a = initial concentration of dehydroascorbic acid; x = decrease in concentration after time t; k = velocity constant), it was found that for a given H^+ concentration the values of k were constant. The average values were: in *N* HCl, $k = 0{\cdot}0053$ min.$^{-1}$; in 0·5 *N* HCl, $k = 0{\cdot}0031$ min.$^{-1}$; in 0·2 *N* HCl, $k = 0{\cdot}0015$ min.$^{-1}$; in 0·1 *N* HCl, $k = 0{\cdot}0010$ min.$^{-1}$.

Since the conversion depends on the H^+ concentration the reaction is pseudo-unimolecular. It may also be added that so far the calibration used for the above determinations is based on the supposition that the conversion of dehydroascorbic acid to diketogulonic acid was complete. If, however, equilibrium is reached as described by Herbert *et al.* [1933] the above figures will represent the sum of the velocity constants of the direct and reverse reactions.

The action of H_2S on diketogulonic acid solutions. When the mutarotation of dehydroascorbic acid reaches completion, treatment of the resulting solution with H_2S yields an amount of ascorbic acid corresponding to about 10 % of the original content of dehydroascorbic acid. Herbert *et al.* [1933] suggested that this is due to an equilibrium between diketogulonic acid and dehydroascorbic acid. This hypothesis has been questioned by Borsook, Davenport, Jeffreys & Warner [1937] who found that by reducing a mutarotated solution of dehydroascorbic acid with glutathione at *p*H 7 reconversion of the diketogulonic acid did not take place as would have been expected in true equilibrium solution.

It was of interest to follow the mutarotation of a dehydroascorbic acid solution by determining the diketogulonic acid formed directly, by means of the colorimetric method and at the same time indirectly by difference after reducing the mutarotating solution with H_2S (on the assumption that the subsequent indophenol reduction was due to the formation of ascorbic acid from the dehydroascorbic acid of the mutarotating solution). Fig. 4 gives a graphic

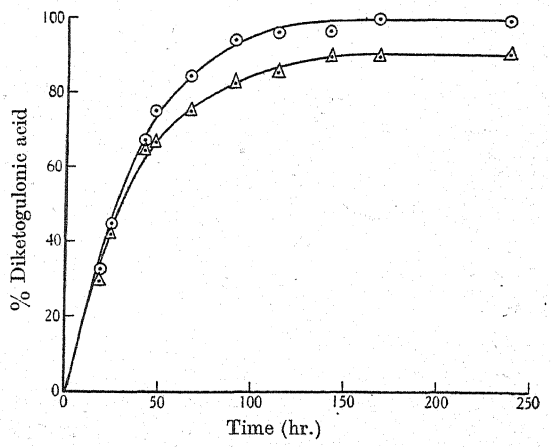
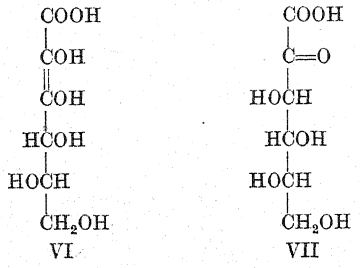


Fig. 4. The course of the mutarotation of dehydroascorbic acid at 25° as determined by the colorimetric and indirect H_2S reduction methods. ⊙——⊙ Colorimetric method. △——△ Indirect H_2S reduction method.

representation of an experiment in which the dehydroascorbic acid present (100 mg./ml.) was obtained by oxidation of a solution of ascorbic acid with I_2. The difference between the results obtained by the two methods up to about 70 % conversion falls within the limits of the experimental error.

After this a deviation eventually reaching a difference of about 10 % was noted. This difference was to be expected, since, in the colorimetric determination, it was assumed that the conversion into diketogulonic acid was complete. An observation was, however, made in the course of this experiment which suggests that the change produced by H_2S in a mutarotated solution is rather more complex than might at first be expected. When diketogulonic acid was determined colorimetrically in the mutarotated solution after H_2S treatment a very low value, about 20 % of that expected, was obtained. Apparently some chemical change in the diketogulonic acid had occurred on treatment with H_2S. The conversion of diketogulonic acid (III) to a tautomeric form (VI) of 2-keto-*l*-gulonic acid (VII), a compound capable of conversion into ascorbic acid [Reichstein & Grüssner, 1934], is one of the changes that can be visualized.



If this scheme be correct the presence of 10 % dehydroascorbic acid in equilibrium with 90 % diketogulonic acid need not therefore be postulated, and consequently the quantity of diketogulonic acid determined by the colour reaction may indeed indicate a 100 % conversion. Whatever be the case the colorimetric method is applicable to the experiments to be described later, since mutarotated solutions which attained 'equilibrium' were employed in the calibration.

Influence of p*H on the mutarotation of dehydroascorbic acid at 38°.* It has already been shown that the concentration of acid in the medium influenced the rate of mutarotation. In the present experiments the mutarotation of dehydroascorbic acid was studied at higher *p*H values. The ascorbic acid was oxidized with I_2 and the solution was brought to 38° as rapidly as possible. 5 ml. dehydroascorbic acid solution were then added to each of a series of tubes containing 10 ml. McIlvaine's phosphate-citrate buffer and 20 ml. H_2O also at 38°. All buffer salts used in these experiments were recrystallized twice from glass-distilled water. For the determination of diketogulonic acid colorimetrically as well as indirectly by H_2S reduction, the tubes were removed at various intervals, cooled, and the contents transferred to a 50 ml. graduated flask, adjusted to *p*H 2 by addition of HCl, and made up to volume.

It will be seen from Fig. 5 that the rate of mutarotation as measured colorimetrically was markedly accelerated by an increase in *p*H. Whilst at *p*H 7·4 the maximum conversion of about 80 % was attained in less than 15 min., only 30 % conversion was recorded after 300 min. at *p*H 4. It may be

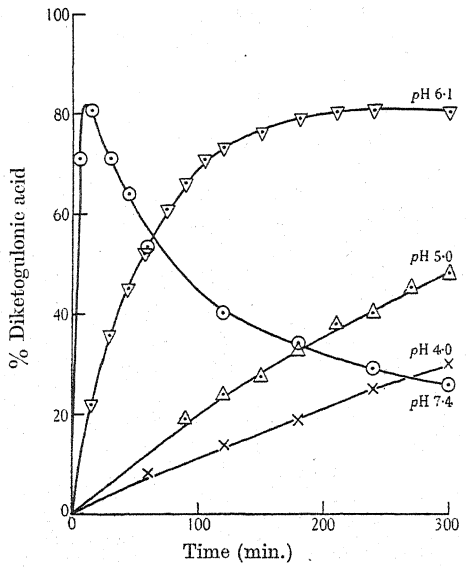


Fig. 5. The influence of *p*H on the rate of mutarotation of dehydroascorbic acid at 38°.

mentioned that at *p*H 4 or 5 the figures obtained by the two methods were almost identical, as would be expected from our previous observations (Fig. 4), since not more than 50 % of the dehydroascorbic acid had been converted. It is also of interest to note that whilst at *p*H 4 and at *p*H 5 the mutarotation proceeds normally as a reaction of the first order, this is not true at *p*H 6·1. Above *p*H 6 it was found that diketogulonic acid began to decompose, the rate of disappearance increasing with increasing *p*H. The observation that maximum conversion of only 80 % could be obtained at *p*H values greater than 6 could to some extent be explained by the decomposition of the diketogulonic acid. Borsook *et al.* [1937], employing electrometric methods at 35·5°, concluded that at *p*H 4·0–5·75 the mutarotation was a reaction of the first order, but at *p*H 6·43 the results obtained were too complicated for interpretation. This, as in our experiments, may have been due to the disappearance of diketogulonic acid at the latter *p*H. Ball [1937], using an electrometric technique which promoted the rapid establishment of the electrode potential, found the reaction at 30° to be of the first order between the *p*H values of 5·188 and 8·566. These results do not appear to agree completely with those obtained in our experiments and those of

Borsook *et al.*; since Ball used a technique involving rapid adjustment of electrode potential, a comparison is not strictly justified.

The discrepancy between the figures obtained by the colorimetric method and by indirect H_2S reduction at *p*H 7·4 in McIlvaine buffer is shown in Fig. 6. It will be seen that when the diketogulonic

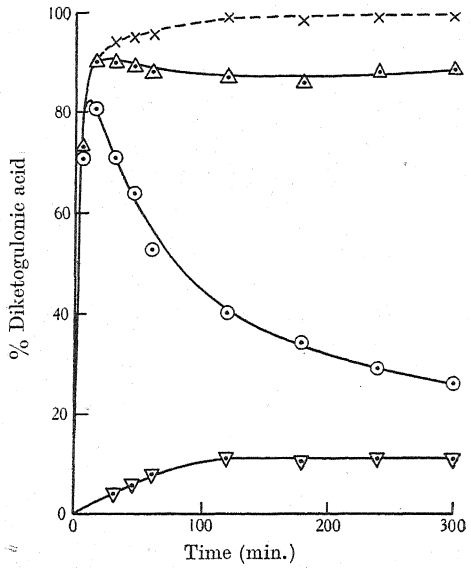


Fig. 6. The course of the mutarotation of dehydroascorbic acid at *p*H 7·4 (McIlvaine's phosphate-citrate buffer) and at 38°. ⊙——⊙ Diketogulonic acid as determined colorimetrically. △——△ Diketogulonic acid as determined indirectly by H_2S reduction. ▽——▽ Indophenol-reducing capacity before H_2S reduction. ×---× Diketogulonic acid as determined indirectly by H_2S reduction after correcting for initial indophenol-reducing capacity.

acid was determined directly a maximum value of about 80 % was obtained in less than 15 min., after which time the diketogulonic acid content began to decrease, attaining a value of about 25 % after 300 min. The indirect method of determining diketogulonic acid at this *p*H failed completely. Under these conditions the production of a substance which reduced indophenol without treatment with H_2S was observed. The reducing power gradually increased and eventually attained a maximum value. After treatment with H_2S the indophenol-reducing capacity was only slightly increased as will be seen from Fig. 6, in which values corrected for the initial reducing capacity are represented by an interrupted line.

That the rate of mutarotation at *p*H 4, 5 and 6 was affected by the *p*H of the solution *per se* and not by the constituents of the buffers, was indicated by the fact that identical results were obtained when phthalate buffer was used instead of McIlvaine phosphate-citrate buffer. At *p*H 7·4, when phosphate-NaOH buffer was used, the conversion of

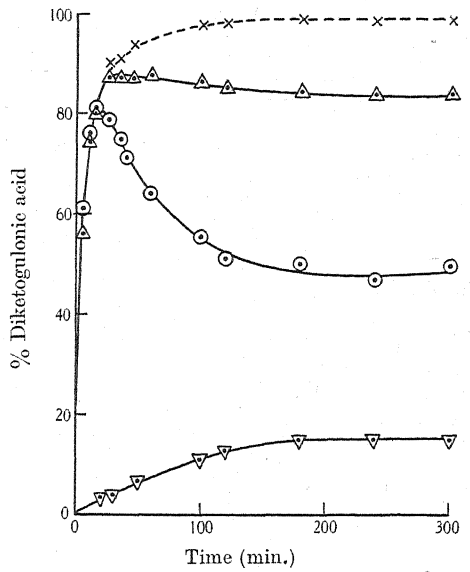


Fig. 7. The course of the mutarotation of dehydroascorbic acid at *p*H 7·4 (phosphate-NaOH buffer) and at 38°. Symbols as in Fig. 6.

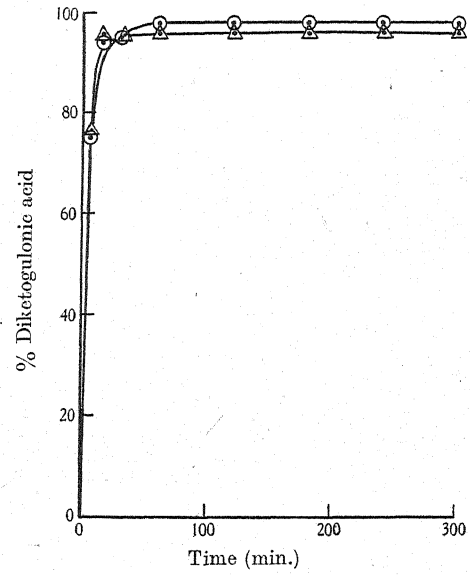


Fig. 8. Influence of borate buffer on the mutarotation of dehydroascorbic acid at *p*H 7·4 and at 38°. Symbols as in Fig. 6.

dehydroascorbic acid into diketogulonic acid and the initial decomposition of the latter proceeded as in the presence of McIlvaine buffer. After about

150 min., however, when the value recorded for diketogulonic acid was 50%, the reaction became unreliable as the colour characteristic of diketogulonic acid was no longer obtained (Fig. 7). The production of an indophenol-reducing substance was also observed when this buffer was used, the indophenol-reducing capacity again being only slightly increased after H_2S treatment. In the presence of borate buffer (*p*H 7·4), almost complete conversion to diketogulonic acid occurred, and good agreement was found between results obtained by the direct colorimetric method and the indirect H_2S reduction method (Fig. 8). The resulting diketogulonic acid was relatively stable and no indophenol-reducing substance was formed from the mutarotating solution. It is of interest to note that diketogulonic acid solution, when added to borate buffer at *p*H 7·4, did not decompose. Since borate is known to combine with polyhydroxy compounds, it might be expected to have a stabilizing effect on the diketogulonic acid.

equivalent of 2·5 mg. ascorbic acid was placed in each stopper. The tubes were then evacuated, washed with O_2-free nitrogen, re-evacuated and the contents mixed after they had attained the temperature of the bath. After various intervals the *p*H was adjusted to 2 and the diketogulonic acid determined by the colorimetric method as well as by the indirect H_2S reduction method.

A control experiment was carried out in air. The results are graphically represented in Fig. 9. It is seen at once that the course of the conversion of the dehydroascorbic acid into diketogulonic acid as measured colorimetrically was the same in both cases. Furthermore, the subsequent change in the diketogulonic acid formed in both experiments was similar to that already observed. On the other hand, the indophenol-reducing capacity of the mutarotated solution before and after H_2S treatment when carried out in the absence of air differed significantly from that observed under aerobic conditions. No indophenol-reducing substance was

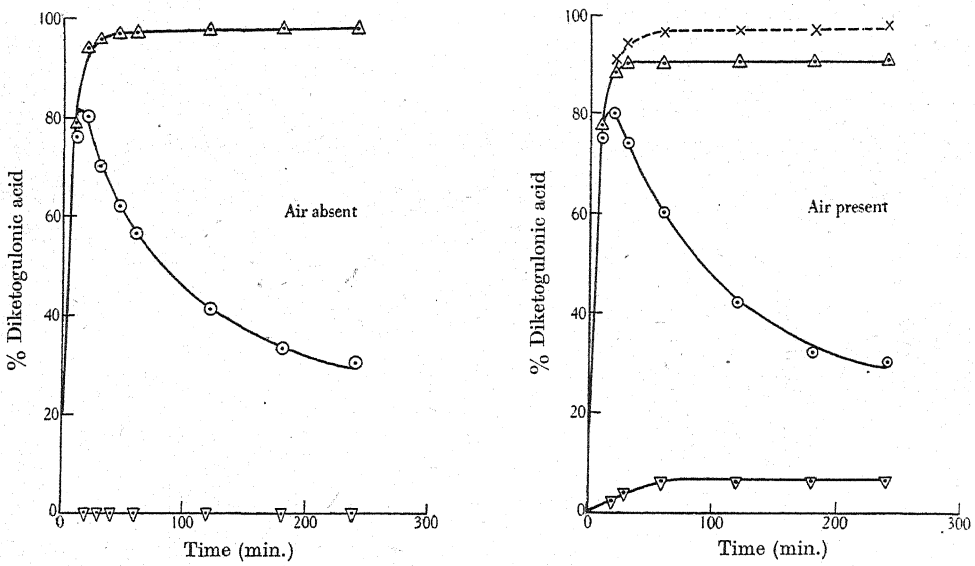


Fig. 9. The influence of O_2 on the mutarotation of dehydroascorbic acid at *p*H 7·4 (McIlvaine's phosphate-citrate buffer) and at 38°. Symbols as in Fig. 6.

Mutarotation of dehydroascorbic acid at p*H 7·4 in the presence and absence of air.* The mutarotation of dehydroascorbic acid at *p*H 7·4 in the absence of air was determined in a series of evacuated modified Thunberg tubes maintained at 38°. The dehydroascorbic acid was prepared by oxidizing a solution of ascorbic acid in oxygen-free water with I_2 and neutralizing the HI formed with NaOH. McIlvaine's buffer was also prepared in oxygen-free H_2O and was adjusted to *p*H 7·4 electrometrically. 5 ml. buffer was introduced into each of the tubes and 1 ml. dehydroascorbic acid solution containing an

produced during the course of the anaerobic experiment, and after treatment with H_2S the indophenol titration was very low. This latter reduction was of the same order as the increase found after H_2S treatment when mutarotation was allowed to proceed in the presence of air. This observation offers further evidence concerning the unreliability of determining diketogulonic acid indirectly by H_2S reduction.

Effect of temperature on the mutarotation of dehydroascorbic acid. The mutarotation of dehydroascorbic acid in the presence of HI formed by the

oxidation of ascorbic acid by I_2 (100 mg./100 ml.) was studied colorimetrically at 25 and 38°. The conversion, as would be expected, proceeded at a higher rate at 38° than at 25° (Fig. 10).

mutarotate for 14 days at room temperature. After this time the conversion of dehydroascorbic acid to diketogulonic acid, as assessed colorimetrically and by H_2S reduction, was complete. This solution was

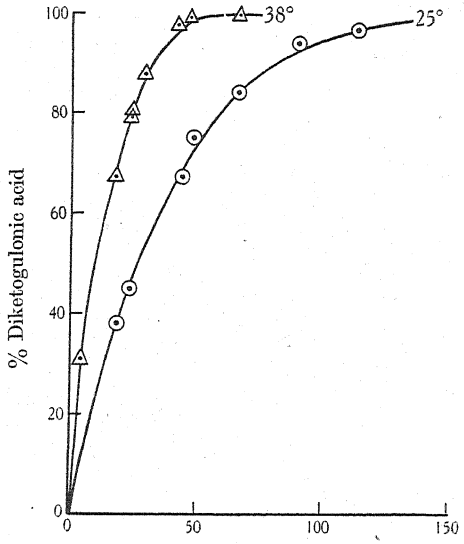


Fig. 10.

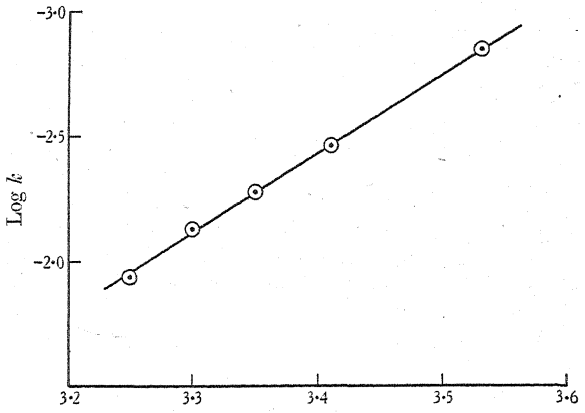


Fig. 11.

Fig. 10. Influence of temperature on the rate of mutarotation of dehydroascorbic acid in the presence of 0·011 *N* HI.

Fig. 11. Influence of temperature on the rate of mutarotation of dehydroascorbic acid in *N* HCl.

In addition, the production of diketogulonic acid was determined in *N* HCl at 10, 20, 25, 30 and 35°. The results when substituted in the formula of a reaction of the first order gave a constant for each temperature. In Fig. 11 $\log k$ is plotted against $1/T$ according to the usual integrated form of the Arrhenius equation, and it will be seen that a linear relationship exists. The temperature coefficient k_{35}/k_{25} calculated from the above data is 2·2.

II. EXPERIMENTS *IN VIVO*

The biological experiments consisted of the determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid in the tissues, in the contents of the digestive tract and in the urine at various intervals after the administration of the last two compounds *per os* or parenterally to guinea-pigs weighing 300–350 g.

The dehydroascorbic acid employed in these experiments was prepared immediately before administration by the oxidation of ascorbic acid with the theoretical quantity of KIO_3. Diketogulonic acid was obtained by allowing a solution of dehydroascorbic acid, adjusted to *p*H 2 with HCl, to partially neutralized with NaOH immediately before use. Equivalents of 120 mg. ascorbic acid contained in approximately 4 ml. were used in each experiment. As the volume of the dose was high it was found convenient, when injecting, to introduce equal parts successively into each of the femoral and biceps muscles of the animals. All the guinea-pigs used in this investigation were previously kept for 5 days on a scorbutic diet to ensure depletion of ascorbic acid in their tissues, so that any formation of ascorbic acid after the administration of dehydroascorbic acid or diketogulonic acid could be detected. After administration of the dose, the animals were placed in metabolism cages for 15, 30 or 60 min., so that any urine voided could be collected for analysis. The animals were then killed after the appropriate time by stunning and bleeding, the blood being collected in oxalate. The tissues and the contents of the intestinal tract were then prepared for analysis as follows. The plasma obtained after centrifuging the blood was precipitated with an equal volume of 10% $CCl_3.COOH$. The contents of the stomach and intestines were obtained by ligaturing the digestive tract at the appropriate places, removing the contents by washing

out with water, making up to a given volume, and finally extracting with an equal volume of 10% CCl_3.COOH. The stomach and intestinal tissues, after drying between filter paper, and also the kidneys and liver were extracted with two parts by weight of 10% CCl_3.COOH. Urine, if present, was removed from the bladder by means of a Pasteur pipette and, if necessary, added to that passed before death. The body was then skinned and the 'carcass' was prepared for analysis as described by Zilva [1935]. After removal of the hair, the skin was minced thoroughly and a sample was extracted with two parts by weight of 10% CCl_3.COOH. Ascorbic acid and dehydroascorbic acid were then determined by titration with indophenol, the former before, and the latter after, H_2S treatment. Diketogulonic acid was assessed by a modified form of the colorimetric method already described by us [Penney & Zilva, 1943]. We found that the sensitivity of the original method could be increased by partial neutralization of the acid present in the test solution immediately after the addition of the dinitrophenylhydrazine reagent. A calibration curve was therefore constructed for this purpose by adding 0·7 ml. 2*N* $KHCO_3$ to the standard after the addition of 1 ml. dinitrophenylhydrazine. The test solutions were treated similarly, allowing for the presence of the CCl_3.COOH acid in the extracts. The standard error of percentage deviation of this modified method was 10%. This modification was not necessary in the case of urine and plasma because in these instances higher values were under consideration. The solutions obtained in the determination of diketogulonic acid in the 'carcass' were turbid; a blank reading was obtained immediately after the addition of the dinitrophenylhydrazine reagent to the extract and subsequent treatment with alkali, and used as a correction to the final reading.

The average quantities of ascorbic acid, dehydroascorbic acid and diketogulonic acid present in the tissues, stomach and intestinal contents and urine 15, 30 and 60 min. after the administration of dehydroascorbic acid or diketogulonic acid *per os* or by injection are given in Tables 5–8, together

Table 5. *Effect of injection of 120 mg. dehydroascorbic acid into the guinea-pig*

		Quantity found													
		Liver			Stomach		Small intestine		Large intestine				Excreted in		
After min.		Total mg.	mg./g.	Kidney mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	'Carcass' mg.	Skin mg.	urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	11·4	0·91	0·99	0·35	0·0	2·2	0·1	1·3	0·0	12·6	4·2	0·0	0·05	33·8
	S.D.*	1·1	0·1	0·08	0·1	0·0	0·0	0·1	0·1	0·0	3·1	1·0	0·0	0·01	5·1
							Dehydroascorbic acid								
	Mean	0·1	0·01	0·05	0·01	0·0	0·0	0·0	0·0	0·2	8·9	1·7	0·2	0·0	11·1
	S.D.	0·1	0·01	0·05	0·01	0·0	0·0	0·0	0·0	0·3	2·3	1·0	0·1	0·0	3·6
							Diketogulonic acid								
	Mean	2·6	0·20	0·99	0·45	1·0	1·7	1·1	1·7	4·8	21·2	12·7	1·0	0·36	54·6
	S.D.	0·5	0·03	0·07	0·18	0·3	0·8	0·2	0·4	2·3	4·7	1·0	0·5	0·05	6·7
							Ascorbic acid								
30	Mean	14·8	1·16	1·09	0·36	0·0	2·4	0·5	1·4	1·0	9·6	3·7	0·1	0·06	35·8
	S.D.	1·1	0·07	0·35	0·06	0·0	0·3	0·1	0·1	0·9	1·7	1·1	0·1	0·02	2·3
							Dehydroascorbic acid								
	Mean	0·2	0·01	0·00	0·04	0·0	0·0	0·1	0·0	0·0	4·6	0·3	0·6	0·00	5·8
	S.D.	0·3	0·01	0 00	0·02	0·0	0·0	0·1	0·0	0·0	0·9	0·3	0·4	0·00	0·8
							Diketogulonic acid								
	Mean	3·3	0·26	1·05	0·46	0·6	1·1	0·9	1·5	5·9	17·5	10·6	3·2	0·29	50·4
	S.D.	0·5	0·06	0·16	0·11	0·2	0·3	0·4	0·3	0·8	5·6	1·0	1·6	0·01	6·2
							Ascorbic acid								
60	Mean	14·9	1·10	0·99	0·41	0·1	3·3	0·6	1·5	0·7	10·8	4·2	0·6	0·07	35·9
	S.D.	1·8	0·11	0·09	0·04	0·1	0·2	0·3	0·2	0·7	2·3	—	0·6	0·02	3·2
							Dehydroascorbic acid								
	Mean	0·0	0·00	0·00	0·02	0·0	0·0	0·1	0·0	0·7	1·4	0·6	0·8	0·00	3·0
	S.D.	0·0	0·00	0·00	0·02	0·0	0·0	0·1	0·0	0·7	2·5	—	0·5	0·00	2·2
							Diketogulonic acid								
	Mean	3·4	0·25	0·88	0·37	0·5	1·1	1·1	1·4	5·0	12·7	16·8	9·1	0·17	42·5
	S.D.	1·0	0·09	0·01	0·10	0·1	0·1	0·0	0·3	0·9	8·5	—	6·6	0·01	11·0

In this and in the following tables, it was assumed that the total volume of plasma from each guinea-pig was 15 ml.

* Standard deviation.

Table 6. *Effect of oral administration of 120 mg. dehydroascorbic acid to the guinea-pig*

After min.		Quantity found: Liver Total mg.	Liver mg./g.	Kidney mg.	Stomach Tissue mg.	Stomach Contents mg.	Small intestine Tissue mg.	Small intestine Contents mg.	Large intestine Tissue mg.	Large intestine Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	3·5	0·27	0·30	0·47	1·0	5·1	4·0	0·9	0·4	4·7	1·5	0·0	0·03	22·5
	S.D.	0·4	0·1	0·05	0·08	0·2	0·5	0·9	0·1	0·4	0·3	0·4	0·0	0·01	1·8
							Dehydroascorbic acid								
	Mean	0·1	0·01	0·03	0·59	51·5	0·4	3·2	0·0	0·0	1·7	0·6	0·0	0·00	58·0
	S.D.	0·1	0·01	0·03	0·03	3·9	0·3	2·6	0·0	0·0	1·7	0·4	0·0	0·00	6·5
							Diketogulonic acid								
	Mean	2·6	0·19	0·41	0·41	2·3	3·5	15·8	1·3	9·8	5·5	4·9	0·1	0·05	45·8
	S.D.	0·9	0·03	0·02	0·06	2·1	0·4	9·7	0·1	2·0	1·6	1·7	0·1	0·01	11·5
							Ascorbic acid								
30	Mean	3·5	0·30	0·45	0·48	0·9	4·7	2·8	1·1	0·5	6·1	2·7	0·1	0·05	24·1
	S.D.	0·6	0·05	0·18	0·12	0·5	0·9	1·6	0·2	0·8	2·9	1·2	0·1	0·02	5·9
							Dehydroascorbic acid								
	Mean	0·3	0·02	0·01	0·16	45·8	0·0	2·1	0·0	0·2	1·0	0·5	0·01	0·00	50·2
	S.D.	0·1	0·01	0·01	0·18	18·1	0·0	0·8	0·0	0·4	0·9	0·1	0·01	0·00	16·5
							Diketogulonic acid								
	Mean	1·9	0·17	0·37	0·40	3·3	1·9	12·2	1·2	13·1	6·6	5·9	0·7	0·05	48·4
	S.D.	0·1	0·01	0·09	0·06	0·2	0·7	3·0	0·4	10·6	1·9	2·0	0·4	0·01	9·2
							Ascorbic acid								
60	Mean	5·6	0·37	0·49	0·40	0·2	5·0	3·2	1·2	0·8	5·0	2·5	0·1	0·04	25·1
	S.D.	1·4	0·02	0·17	0·11	0·3	1·4	0·6	0·2	0·8	0·7	1·2	0·1	0·01	5·5
							Dehydroascorbic acid								
	Mean	0·2	0·01	0·01	0·06	5·0	0·2	1·2	0·0	0·0	1·0	0·6	0·0	0·00	8·2
	S.D.	0·2	0·01	0·01	0·03	1·5	0·2	0·3	0·0	0·0	0·7	0·6	0·0	0·00	2·2
							Diketogulonic acid								
	Mean	2·4	0·14	0·37	0·26	1·5	2·2	14·2	1·5	18·8	2·7	5·9	0·8	0·04	51·3
	S.D.	0·6	0·03	0·05	0·02	0·4	0·9	5·6	0·2	3·1	2·4	0·6	0·5	0·01	9·5

Table 7. *Effect of injection of 120 mg. diketogulonic acid into the guinea-pig*

After min.		Quantity found: Liver Total mg.	Liver mg./g.	Kidney mg.	Stomach Tissue mg.	Stomach Contents mg.	Small intestine Tissue mg.	Small intestine Contents mg.	Large intestine Tissue mg.	Large intestine Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	0·9	0·08	0·08	0·11	0·0	0·8	0·0	0·5	0·0	1·8	0·6	0·0	0·00	4·8
	S.D.	0·1	0·01	0·01	0·02	0·0	0·2	0·0	0·1	0·0	0·2	0·2	0·0	0·00	0·3
							Diketogulonic acid								
	Mean	2·6	0·20	1·19	0·48	0·7	0·9	0·9	1·3	3·8	43·4	16·0	7·5	0·55	86·6
	S.D.	0·5	0·01	0·06	0·06	0·1	0·1	0·1	0·3	0·7	0·3	1·0	1·1	0·03	2·3
							Ascorbic acid								
30	Mean	1·0	0·07	0·09	0·08	0·0	0·9	0·0	0·5	0·0	1·4	0·6	0·0	0·00	4·5
	S.D.	0·3	0·01	0·00	0·02	0·0	0·2	0·0	0·1	0·0	0·8	0·2	0·0	0·00	1·3
							Diketogulonic acid								
	Mean	3·2	0·21	1·28	0·43	0·9	1·0	0·7	1·4	4·7	26·6	15·4	11·5	0·45	73·8
	S.D.	0·7	0·01	0·12	0·08	0·6	0·2	0·2	0·3	1·6	4·3	1·0	5·5	0·01	9·6
							Ascorbic acid								
60	Mean	0·9	0·07	0·10	0·09	0·0	0·9	0·0	0·6	0·0	1·1	0·6	0·1	0·00	4·3
	S.D.	0·2	0·01	0·03	0·01	0·0	0·1	0·0	0·1	0·0	0·8	0·3	0·2	0·00	1·0
							Diketogulonic acid								
	Mean	2·3	0·17	0·90	0·35	2·1	1·1	0·6	1·2	4·6	11·7	8·9	9·3	0·23	46·5
	S.D.	0·4	0·02	0·17	0·06	0·5	0·1	0·1	0·1	0·9	8·4	2·7	7·2	0·05	18·1

No dehydroascorbic acid was detected in this experiment.

Table 8. *Effect of oral administration of 120 mg. diketogulonic acid to the guinea-pig*

After min.		Quantity found: Liver Total mg.	Liver mg./g.	Kidney mg.	Stomach Tissue mg.	Stomach Contents mg.	Small intestine Tissue mg.	Small intestine Contents mg.	Large intestine Tissue mg.	Large intestine Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	0·6	0·05	0·07	0·12	0·0	1·0	0·1	0·4	0·0	1·6	0·5	0·0	0·00	4·1
	S.D.	0·2	0·01	0·01	0·02	0·0	0·2	0·1	0·1	0·0	0·1	—	0·0	0·00	0·3
							Diketogulonic acid								
	Mean	2·2	0·17	0·34	0·91	55·6	4·2	18·5	1·0	4·8	2·8	6·6	0·7	0·05	94·0
	S.D.	0·3	0·04	0·04	0·08	10·7	0·7	1·5	0·2	0·6	2·0	—	0·6	0·01	14·4
							Ascorbic acid								
30	Mean	0·6	0·05	0·07	0·12	0·0	1·5	0·2	0·6	0·2	0·9	0·3	0·0	0·00	4·5
	S.D.	0·2	0·01	0·03	0·01	0·0	0·2	0·2	0·1	0·3	0·8	0·2	0·0	0·00	1·8
							Diketogulonic acid								
	Mean	1·8	0·16	0·32	0·89	23·3	3·7	9·8	2·4	13·7	7·3	5·6	1·1	0·07	70·5
	S.D.	0·3	0·01	0·07	0·17	11·0	2·3	9·3	1·3	4·7	4·2	0·6	0·2	0·02	13·1
							Ascorbic acid								
60	Mean	0·7	0·06	0·08	0·13	0·0	1·4	0·6	0·7	0·5	1·5	0·5	0·0	0·00	6·1
	S.D.	0·1	0·01	0·02	0·02	0·0	0·2	0·4	0·2	0·5	0·3	0·1	0·0	0·00	0·7
							Diketogulonic acid								
	Mean	2·1	0·18	0·32	0·88	31·0	3·4	14·5	1·9	17·7	0·5	4·5	1·1	0·04	78·4
	S.D.	0·4	0·03	0·02	0·13	4·1	1·5	8·5	0·1	6·7	0·8	1·3	0·2	0·01	1·6

No dehydroascorbic acid was detected in this experiment.

Table 9. *Analysis of tissues from control guinea-pigs*

	Quantity found: Liver Total mg.	Liver mg./g.	Kidney mg.	Stomach Tissue mg.	Stomach Contents mg.	Small intestine Tissue mg.	Small intestine Contents mg.	Large intestine Tissue mg.	Large intestine Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
						Ascorbic acid								
Mean	0·6	0·05	0·04	0·10	0·0	0·6	0·0	0·5	0·0	1·4	0·6	0·0	0·00	3·5
S.D.	0·3	0·02	0·02	0·05	0·0	0·2	0·0	0·1	0·0	0·6	0·0	0·0	0·00	0·5
						Diketogulonic acid								
Mean	1·9	0·17	0·33	0·27	0·6	0·8	0·8	0·9	4·8	4·7	6·1	0·2	0·05	17·9
S.D.	0·2	0·01	0·08	0·02	0·1	0·1	0·3	0·2	1·9	1·8	0·0	0·1	0·01	6·9

No dehydroascorbic acid was detected in this experiment.

with the standard deviations. Each experimental group consists of three guinea-pigs. The concentrations of these substances in the liver are also given in the Tables. The approximate concentrations in the other tissues can be obtained from the mean weights of the organs, which were as follows: kidney 2·75 g., stomach 3·15 g., small intestine 11·0 g., large intestine 11·2 g., 'carcass' 164 g., skin 57 g. All the results are calculated as equivalents of ascorbic acid. A group of animals depleted of vitamin C for 5 days but not receiving either dehydroascorbic acid or diketogulonic acid was analysed and these results were used as controls (Table 9). All the tissues of the control animals gave a slight colour reaction, but in all probability this was due to the presence of substances other than diketogulonic acid. As the experiments were repeated several times and data of a similar trend were obtained, only representative results are given in this communication.

Significance of the biological results

Sufficient evidence has been obtained to elucidate the behaviour of dehydroascorbic acid and diketo-

gulonic acid in the living guinea-pig. When dehydroascorbic acid was introduced into the organism by intramuscular injection it was very soon partly reduced by the organism to ascorbic acid and partly converted to diketogulonic acid. The former entered the tissues. The latter was mainly excreted by the kidneys but some of it was also found deposited in some of the tissues (Table 5). As will be seen from a separate experiment, the results of which are given in Table 10, as much as about 25 % of the quantity of dehydroascorbic acid injected

Table 10. *Excretion of diketogulonic acid in 24 hr. after the injection of dehydroascorbic acid or diketogulonic acid*

Day	Substance injected		Diketogulonic acid excreted in 24 hr. (mg.)		
		Animal no. ...	41	42	43
1	None		5·6	6·0	5·8
2	120 mg. dehydroascorbic acid		32·6	21·6	38·8
3	None		4·1	8·4	8·3
		Animal no. ...	44	45	46
1	None		5·8	8·7	8·2
2	120 mg. diketogulonic acid		23·5	36·8	35·1
3	None		7·6	11·7	7·2

can be excreted as diketogulonic acid in 24 hr., mostly during the first 3 or 4 hr. Similar results were obtained when diketogulonic acid was injected. Dehydroascorbic acid was estimated at the same time, and the results indicated that possibly small quantities of dehydroascorbic acid were excreted. The identity of diketogulonic acid in the urine after injection of dehydroascorbic acid was established by isolating and identifying the dinitrophenylhydrazine derivative as described previously. The crude yield was in large excess of that which would have been expected from the ascorbic acid present and was in fair agreement with the total quantity of ascorbic acid and diketogulonic acid found in the urine. M.P. after recrystallization from acetone-ethanol was 278°. Mixed M.P. with authentic derivative was 279°. N content (Weiler) = 20·7 % (calculated for osazone N = 21·0 %).

It will be seen from Table 5 that the concentration of ascorbic acid in the liver after injection of 120 mg. dehydroascorbic acid was very high (about 1 mg./g.). When 120 mg. ascorbic acid were injected we found that the ascorbic acid concentration was only about one-half this value. It appears that most if not all the unchanged dehydroascorbic acid which entered the blood stream and which had not mutarotated was reduced in the liver, since none was found in the blood after administration. Therefore when dehydroascorbic acid is injected the concentration of ascorbic acid in the blood is not the only factor which controls the concentration in the liver.

When dehydroascorbic acid was administered *per os* (Table 6) it passed gradually into the system but part of it remained almost entirely unchanged in the stomach before being absorbed. About 50 % could be recovered as dehydroascorbic acid after 15 min. The dehydroascorbic acid in the stomach decreased with time, by absorption and passage into the small intestine, where, owing to the prevailing alkaline condition, it was converted to diketogulonic acid. No dehydroascorbic acid was found under the conditions of this experiment to reach the caecum, colon or rectum.

When diketogulonic acid was given *per os* (Table 8) it was found that most of the dose passed from the stomach along the intestine and no doubt would eventually have been excreted in the faeces either unchanged or as decomposition products. Since small quantities were found in the tissues of the digestive tract and in the urine, it may be presumed that minute quantities had passed into the blood stream although the presence of diketogulonic acid could not be demonstrated in the plasma. This may be explained by the fact that the little that entered the blood was excreted by the kidneys as fast as it passed through the intestinal tissues. Furthermore, as will be seen later, diketogulonic acid undergoes chemical change in the animal tissues. When the compound was injected the greater part was found in the 'carcass', skin and plasma (Table 7). Significant increases were also noted in the kidneys. 15 min. after injection the total amount found in the body of the animal was about 70 mg.; this quantity decreased with time and after 60 min. the amount recovered was about 30 mg., up to 15 mg. of which was found in the urine. No doubt diketogulonic acid is changed chemically in the body, as it is *in vitro* at *p*H 7·4 at 38° (cf. Fig. 9). Whether the compound was administered orally or by injection, no evidence was obtained indicating any significant lactonization of diketogulonic acid. Doubtful traces of ascorbic acid have been observed in some of the tissues and in the urine after the administration of diketogulonic acid. Their presence could be explained by the fact that mutarotated solutions were used, and these are known to yield small quantities of ascorbic acid when reduced by H_2S. Even if these minute amounts were derived from diketogulonic acid very large quantities of this open-chain compound would have to be administered in order to exert any antiscorbutic effect. It is therefore not surprising that Hirst & Zilva [1933] and Borsook *et al.* [1937] had observed no antiscorbutic effect when employing small doses of mutarotated dehydroascorbic acid.

Consideration of the total recovery of ascorbic acid and dehydroascorbic acid only after the ad-

ministration of dehydroascorbic acid shows that a great part of the dose is not accounted for. This, as can now be seen, is due to the fact that considerable quantities of dehydroascorbic acid are converted to diketogulonic acid. The *in vitro* experiments (cf. Fig. 9) explain the reason for this rapid conversion. At *p*H 7·4 and 38° the conversion of a great part of the dehydroascorbic acid into diketogulonic acid can take place within the first few minutes. As already mentioned it was observed in the preliminary experiments which led to this investigation that whilst most of the ascorbic acid could be accounted for in the body soon after injection this was not the case when dehydroascorbic acid was injected, and it was then suspected that part of the latter was converted into diketogulonic acid. This hypothesis is now fully supported by experimental evidence.

DISCUSSION

The main object of this investigation was to obtain experimental evidence which might offer some support to the hypothesis concerning the mode of action of ascorbic acid as outlined in the introduction. It was assumed that ascorbic acid was a link in a co-ordinated chain of reactions, in which it was alternately oxidized to dehydroascorbic acid and reduced to its original form. The presence of the reversibly oxidized product in minute quantities as a component available for further interaction was postulated accordingly. It is of interest now to consider how our results can be co-ordinated with this hypothesis.

Our *in vitro* and *in vivo* experiments have shown that dehydroascorbic acid tends to be converted to diketogulonic acid with comparative ease both in the presence and in the absence of O_2, the rate being influenced by the *p*H of the medium. On the above hypothesis therefore the reactions would proceed according to the following scheme:

Ascorbic acid $\rightleftharpoons$ dehydroascorbic acid
$\rightarrow$diketogulonic acid.

Our present evidence and that of Hirst & Zilva [1933] and of Borsook *et al.* [1937] does not favour the view that diketogulonic acid can be reconverted to ascorbic acid in the body. This is accomplished *in vitro* only by such drastic methods as treatment with HI [Herbert *et al.* 1933]. It is, however, considered that the concentration of dehydroascorbic acid must at any given time in the above reaction be extremely low, and in consequence the diminution in the quantity of the ascorbic acid concerned through the conversion of dehydroascorbic acid into diketogulonic acid would be very slow. This may account for the long time that elapses before the appearance of scurvy. In animals not capable of synthesizing the vitamin and at the same time not receiving any in the diet, the total content of the ascorbic acid of the body, after the tissues have been depleted, is very small, yet a considerable time has to elapse after the depletion before even latent scurvy can be diagnosed. The daily ascorbic acid lost during this period must therefore be so minute as to fit in with the requirements of the above hypothesis. Admittedly our results indicate no more than a bare possibility of the existence of the scheme outlined above, but they do in our opinion definitely indicate that possibility.

Another point of theoretical interest deserves mention. It was observed that when large doses of dehydroascorbic acid were given orally an increase in ascorbic acid in the tissues of the stomach was found, thus showing that dehydroascorbic acid was reduced there while passing into the blood stream. This observation offers an explanation for the economic utilization of small doses of dehydroascorbic acid. Demole [1933] and Hirst & Zilva [1933] found that dehydroascorbic acid was somewhat less active than ascorbic acid. The latter workers compared the biological response of guinea-pigs on a scorbutic diet receiving a daily oral dose of 0·25, 0·5 and 1 mg. dehydroascorbic acid with that of guinea-pigs receiving similar doses of ascorbic acid. The response of the former group was only very slightly less than that of the latter, and this was noticeable only in the two lower doses. Our present experiments suggest that doses of dehydroascorbic acid of the above order would pass quickly through the stomach wall where they would be converted into ascorbic acid. Dehydroascorbic acid in small doses would therefore begin functioning as ascorbic acid within a short time and any loss through conversion to diketogulonic acid would in consequence be very small. Large doses of dehydroascorbic acid, on the other hand, might be delayed in the stomach or in the small intestine before passing into the blood stream and would thus incur a proportionately higher loss through mutarotation.

The observations made in connexion with the parenteral introduction of dehydroascorbic acid and diketogulonic acid have, as far as we can see at present, no bearing on practical nutrition. The oral consumption of dehydroascorbic acid and even of diketogulonic acid is in all probability encountered in practice. It may occur when the tissues of vegetables, particularly those containing oxidizing enzymes, are disintegrated before being eaten. If large quantities are consumed the dehydroascorbic acid as we have seen may remain for some time in the stomach and in the small intestine before passing into the blood stream. The destruction of the antiscorbutic potency in the case of dehydroascorbic acid would be rather higher than that of ascorbic acid under similar conditions, but since this applies to quantities of vitamin C which are far greater than the physiological requirement it remains a point of theoretical interest only.

SUMMARY

(*a*) In vitro *experiments*

1. Diketogulonic acid in the presence of HCl forms a derivative with dinitrophenylhydrazine. When ascorbic acid and dehydroascorbic acid are present they are first converted to diketogulonic acid which then reacts with the reagent.

2. The percentage of dehydroascorbic acid which is converted to diketogulonic acid in a given time in the presence of mineral acids is independent of the initial concentration of the former and proceeds as a reaction of the first order at any *p*H value below about *p*H 4 investigated by us. The conversion is accelerated by increase in temperature, the temperature coefficient k_{35}/k_{25} being 2·2. At *p*H values higher or lower than about *p*H 4 the rate of conversion is accelerated.

3. Diketogulonic acid undergoes a chemical change in the presence of H_2S. At *p*H 7·4 the rate of formation of diketogulonic acid from dehydroascorbic acid, as measured by the colour reaction, is high and its decomposition proceeds at a considerable rate. This is independent of the presence of air.

The indirect determination of diketogulonic acid by H_2S treatment is not reliable under all conditions.

(*b*) In vivo *experiments*

1. When diketogulonic acid was injected intramuscularly into guinea-pigs which had previously received a scorbutic diet for 5 days, about 60 % of the dose was recovered after 15 min., mainly from the 'carcass', skin and plasma. After 1 hr. only about 30 %, of which as much as one-third was present in the urine, was recovered. It was evidently destroyed in the animal body.

2. When diketogulonic acid was administered orally only small quantities, if any, entered the blood. The greater part of the dose passed from the stomach along the intestine and some no doubt was excreted in the faeces.

3. When dehydroascorbic acid was injected it was partly converted into diketogulonic acid and partly reduced to ascorbic acid, some of which filled the tissues of the unsaturated guinea-pigs and some was excreted by the kidney. The liver contained abnormally high quantities of ascorbic acid. It is suggested that when large quantities of dehydroascorbic acid are introduced into the blood stream reduction takes place mainly in the liver.

4. When dehydroascorbic acid was administered *per os* it was gradually absorbed, but a considerable part of the dose remained at first (15–30 min.) unchanged in the stomach. In the small intestine, owing to alkalinity, it was converted to diketogulonic acid.

5. When 120 mg. diketogulonic acid were injected, about 25 mg. were excreted in 24 hr. The greater part of this passed through the kidney during the first 4 hr. Injected dehydroascorbic acid (120 mg.) was similarly excreted by the kidney as diketogulonic acid, in almost the same quantities.

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Experiments on the Function of Pantothenate in Bacterial Metabolism

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The purpose of this account is to record results obtained in an attempt to determine the function of pantothenate in metabolism. From the observation that pantothenate stimulated O_2-uptake and especially CO_2-output [Williams, Mosher & Rohrmann, 1936; Pratt & Williams, 1939], it was thought that this nutrient might be specifically concerned with the metabolism of α-ketonic acids, since these are an important source, perhaps the only source, of respiratory CO_2. An abstract of earlier results [Hills, 1941] showed that many other substrates may be affected. In order to determine whether this was due to an intermediary metabolite common to these substrates, experiments on interaction between substrates both aerobically and anaerobically have now been carried out, and the course of pyruvate breakdown studied. While the work was in progress an abstract of similar work, with findings differing in some details, was published by Berkman, Dorfman & Koser [1942] who concluded in a later paper [Dorfman, Berkman & Koser, 1942] that pantothenate is concerned with a metabolic step involving pyruvate or a derivative, most likely that of the conversion of pyruvate to acetate. At the same time Teague & Williams [1942] produced evidence that pantothenate was not concerned with glucose phosphorylation, glucose or hexosediphosphate fermentation, or pyruvate decarboxylation. Their test systems were pantothenate-deficient yeast extract and chick brain. Later Pilgrim, Axelrod & Elvehjem [1942] showed that diminished oxidation of pyruvate occurs in pantothenate-deficient rat liver. Although the present study has been confined to a single bacterial species, the results may therefore have wider implications.

METHODS

Organisms. On account of the simplicity of its nutritional requirements, *Proteus morganii* was chosen for this work. Since pantothenate must be supplied in the medium [Pelczar & Porter, 1940], cells deficient in pantothenate, or with sufficient for optimum metabolism, may be obtained at will by regulation of the pantothenate content of the medium. Four strains from the National Collection of Type Cultures, Nos. 1707, 2814A, 2818 and 5845, were found to have similar pantothenate requirements for growth, and a similar deficiency in O_2-uptake when grown with suboptimal amounts. The results quoted refer to strain 2818 except where otherwise stated.

Culture media. The organisms were usually grown on the 'complete' amino-acid medium described by Gladstone [1939], with the addition of 1 g. $(NH_4)_2SO_4$ and 1 g. NH_4Cl/l., and of nicotinamide (10^{-5} *M* final) and synthetic Ca (+) pantothenate (Merck), which were essential nutrients. Good crops of pantothenate-deficient organisms (afterwards described simply as 'deficient' where no ambiguity might arise) were obtained with $2–4 \times 10^{-8}$ *N* pantothenate. Aneurin (10^{-7} *M*) and riboflavin (2×10^{-6} *M*) were sometimes added to avoid partial deficiencies of these factors, but these non-essential additions appeared not to influence the results. Later it was found possible to dispense with all the amino-acids except cystine, which was probably required as a source of organic S [cf. Fildes & Richardson, 1937; Gladstone, 1937]. This NH_3-cystine basal medium was made up similarly to the NH_3 medium of Fildes [1940] with the addition of 25 mg. cystine in NaOH and 1·25 mg. nicotinamide/l. final bulk. Lactate was omitted and glucose added to the basal medium after sterilization. Pantothenate requirements were the same as for the amino-acid medium.

A basal medium consisting of 1 % acid-hydrolysed casein [McIlwain, 1939] has also been used with the addition of glucose, nicotinamide and pantothenate as required. The organisms were not so deficient as those reaped from the synthetic media, even though the pantothenate concentration was reduced to 10^{-8} *M*, the minimum necessary to support adequate growth.

All media were dispensed in 100 ml. quantities in 250 ml. conical flasks. Glucose (1·25 ml. *M*) and pantothenate were added at the time of inoculation. Except where otherwise stated the amino-acid medium was used.

Inoculum. In the early stages, to avoid carrying over pantothenate, a small inoculum of *ca.* 100 organisms/ml. was used but later this proved unsatisfactory. Instead, the inoculum for 100 ml. culture was 1 ml. of an 18–24 hr. culture on the same medium with 10^{-6} *M* pantothenate, which was found to give more reproducible results than cells raked from an agar slope and suspended to give a similar opacity (*ca.* 10^8 cells/ml.). Subcultures were never made from deficient cultures, so as to avoid training the organism to dispense with an added supply of pantothenate.

The inoculated medium was incubated in air for 40–48 hr. with the small inoculum, or for 20–24 hr. with the large inoculum. The cells were collected on the centrifuge and washed once. The yield was *ca.* 10 mg. dry wt. of deficient cells/100 ml. medium, which was enough for 3–5 Warburg vessels in most experiments. Except where stated otherwise the large inoculum was used in the experiments quoted.

Measurement of metabolism. Standard manometric methods as described by Dixon [1934] were used. The cell suspension in distilled water was suitably buffered and added to a salt mixture in Warburg vessels, so that with any additions from the side-bulbs the final composition of the medium was that of Krebs & Henseleit [1932], with replacement of KH_2PO_4 by KCl, and limitation of the Ca^{++} content to $3 \times 10^{-5} N$ including the Ca pantothenate added to certain vessels. This excluded any possible effect on metabolism of the Ca^{++} in the pantothenate, and there were no difficulties due to precipitation when the $NaHCO_3$ buffer was replaced by 30 mM phosphate in the measurement of O_2-uptake by the direct method. Except where otherwise stated the pantothenate concentration was $3 \times 10^{-6} N$. In anaerobic experiments the last traces of O_2 were removed by yellow P in the centre well. The temperature was 37°.

Substrates. Pyruvate was purified by vacuum distillation according to Wendel [1932], and was stored as 5 M solution at 0–5°. It was determined by the carboxylase method according to the details of Westerkamp [1933].

Oxalacetic acid was prepared according to Wohl & Oesterlin [1901] and Wohl & Claussner [1907]. It was crystallized from hot acetone-benzene [Fenton & Jones, 1900] and determined, like acetoacetate, by the aniline citrate method [Edson, 1935; Greville, 1939]. I am indebted to Mr D. E. Hughes for pure preparations of pyruvic and oxalacetic acids. Both these substrates were neutralized just before use with the calculated quantity of $NaHCO_3$.

Except where otherwise stated the final concentration of all substrates was 224 μl. in 3·33 ml. per vessel, i.e. 3 mM (each form where *dl*-mixtures were used).

RESULTS

Pantothenate requirements for growth and metabolism

Table 1 shows the influence of different concentrations of pantothenate in the culture medium on O_2-uptake of the washed cells. The low metabolism of those grown with 1–4 $\times 10^{-8} M$ pantothenate was restored to normal by $3 \times 10^{-6} M$ pantothenate in the final suspension. If we take the yield of organisms into account, however, the amount, in deficient cells, of the enzyme requiring pantothenate was, even when metabolism had been activated subsequently by addition of pantothenate, only 55 % of that produced under optimal conditions. Table 2 shows that the concentration of pantothenate used in the final suspension was ample for optimal activation of both oxidation and fermentation of pyruvate.

Table 1. *Effect of pantothenate concentration during cultivation on O_2-uptake*

Cells grown from a small inoculum. 20 mM pyruvate added after shaking 1 hr. in air.

	Pantothenate (M) during growth		
	8×10^{-9}	4×10^{-8}	10^{-6}
Crop (mg. dry cells/100 ml. medium)	10	21	32
Q_{O_2} (μl./mg. cells):			
No pantothenate	17	16	55
With pantothenate	105	47	56
O_2-uptake (μl./100 ml. culture/hr.):			
No pantothenate	170	340	1760
With pantothenate	1050	990	1790

Table 2. *Effect of added pantothenate on aerobic and anaerobic metabolism of pyruvate by deficient cells*

Strain 2818 grown on hydrolysed casein with $2 \times 10^{-8} M$ pantothenate. The suspensions were shaken for 30 min. in the presence of the stated concentrations of pantothenate before adding pyruvate (20 mM). The data are for the period 40–70 min. after adding the substrate.

Pantothenate added M	Q_{O_2}	$Q^{N_2}_{acid+gas}$
0	54	17
10^{-9}	—	21
3×10^{-9}	59	32
10^{-8}	71	52
3×10^{-8}	81	73
10^{-7}	94	—
10^{-5}	97	73

Influence of pantothenate on oxygen uptake

Specificity of influence of pantothenate. The effect on O_2-uptake was probably specific for pantothenate, since aneurin, hydrolysed pantothenate and a partially purified preparation of cozymase were all ineffective (Table 3). The cozymase preparation stimulated the organism under appropriate conditions (cozymase-deficient cells grown on the hydrolysed casein medium with no added nicotinamide). Hence its ineffectiveness on pantothenate-deficient cells was not likely to be due to its inability to reach the active centres.

Table 3. *Specificity of influence of pantothenate on oxygen uptake of deficient cells*

Strain 2818 grown on pantothenate-deficient medium, i.e. hydrolysed casein with added pantothenate ($10^{-8} M$) and nicotinamide ($10^{-5} M$), and on nicotinamide-deficient medium, i.e. hydrolysed casein with added pantothenate ($10^{-6} M$) and no added nicotinamide.

Addition	Conc. M	Q_{O_2} (20–80 min.) Pantothenate-deficient	Q_{O_2} (20–80 min.) Nicotinamide-deficient
None	—	28	43
Pantothenate	$1{\cdot}5 \times 10^{-7}$	82	45
*Hydrolysed pantothenate	$1{\cdot}5 \times 10^{-6}$	34	—
Aneurin	3×10^{-5}	34	—
Cozymase	2×10^{-6}	35	80

* N HCl 10 min. at 100°.

Substrate specificity. Table 4 shows the effect of pantothenate on the O_2-uptake of deficient cells in the 1st hr. after adding a variety of substrates. The rate of O_2-uptake with pyruvate in the presence of pantothenate is taken as 100, as an arbitrary standard of comparison between different experiments. The total uptake after 4 hr. is given, expressed as mol./mol. substrate supplied. To obtain comparable results in different experiments, the cells from 200 ml. culture were always used for an experiment with 10 vessels. This procedure gave a greater uniformity than that in which a constant dry weight of organisms was used in each vessel. With the exception of glucose, for which the O_2-uptake was not greatly affected by the addition of pantothenate, and of succinate, pyruvate gave a more rapid rate of O_2-uptake than any other substrate tested.

Table 4. *Substrate specificity for influence of pantothenate on oxygen uptake of deficient cells*

To facilitate comparison of substrates with the same C skeleton but different degrees of oxidation, compounds are arranged in order of decreasing number of C atoms and increasing degree of oxidation for a given number of C atoms.

Substrate	Relative rate of O_2-uptake for 1st hr. (uptake with pyruvate and added pantothenate = 100)		Mol. O_2 Taken up in 4 hr.		Theory for completion
	No pantothenate	With pantothenate	No pantothenate	With pantothenate	
(*a*) Using organisms grown from a large inoculum					
Citrate	9	38	0·07	0·38	4·5
dl-Glutamate	17	73	0·15	0·88	4·5*
α-Ketoglutarate	14	55	0·17	0·74	4·0
Succinate	36	104	0·33	1·24	3·5
Fumarate	28	88	0·22	1·06	3·0
l(−) Malate	38	58	0·27	0·63	3·0
dl-Aspartate	20	61	0·19	0·84	3·0*
Oxalacetate	39	80	0·35	1·13	2·5
Glycerol	35	64	1·13	2·14	3·5
d- or *l*-Lactate	33	86	0·24	0·97	3·0
dl-Alanine	17	41	0·15	0·39	3·0*
Pyruvate (10 exp.)	34	100 (Q_{O_2} = 75)	0·36	1·18	2·5
Standard deviation	9	(11)	0·13	0·25	—
None (11 exp.)	6	16	0·04	0·12	—
Standard deviation	3	6	0·03	0·04	—
(*b*) Using organisms grown from a small inoculum					
Glucose	150	166	1·51	2·00	6·0
Galactose	17	33	0·17	0·60	6·0
Butyrate	2	8	0·09	0·14	5·0
β-Hydroxybutyrate	8	14	0·08	0·17	4·5
Acetoacetate	11	14	0·08	0·14	4·0
Succinate	26	36	—	—	3·5
l-Malate	9	17	—	—	3·0
Propionate	4	5	0·06	0·10	3·5
dl-Glyceraldehyde	13	24	0·12	0·24	3·0*
dl-Lactate	31	74	0·37	1·18	3·0
Pyruvate (6 exp.)	39	100 (Q_{O_2} = 51)	0·54	0·86	2·5
Standard deviation	6	(8)	0·10	0·13	—
Ethanol	16	22	0·23	0·39	3·0
Acetaldehyde	7	10	0·19	0·31	2·5
Acetate	8	13	0·15	0·35	2·0
Formate	33	41	0·36	0·52	0·5
None (5 exp.)	7	14	0·07	0·14	—
Standard deviation	4	6	0·03	0·04	—

* Calculated on the assumption that only one optical isomer is oxidized.

At one stage in the work it was found that malate, and to a less extent succinate, gave slower O_2-uptake (Table 4 (*b*)) than was shown by Hills [1941], the lower rate being in agreement with the report of Berkman *et al.* [1942]. Attempts to restore the activity by the addition of animal ash or Mn^{++} to the culture medium [cf. Woolley, 1941], which was suspected to be suboptimal with regard to trace elements, were unsuccessful, but with larger inocula (Table 4 (*a*)) it was found possible to get better growth and an increase in the rate of O_2-uptake in the presence of the C_4-dicarboxylic acids without any special additions to the medium. Since this study had to be abandoned before it was found possible to repeat the work on 12 out of 24 substrates using organisms grown from larger inocula, the data are not all strictly comparable and are divided between two lists: for organisms grown from (*a*) large and (*b*) small inocula respectively. As will be seen later, the latter list probably includes only two substrates, acetate and formate, which may be of importance in the function of pantothenate.

Other substrates which promoted rapid O_2-uptake (50–100 % of the rate with pyruvate) in the presence of pantothenate were: glutamate, α-ketoglutarate, glycerol and both optical isomers of lactate. Galactose, citrate, glyceraldehyde, alanine and formate gave moderate rates of O_2-uptake in the presence of pantothenate, initially 20–50 % of that with pyruvate. With formate, the rate was independent of pantothenate in the first 15 min. but fell off rapidly, more so without added pantothenate. With acetaldehyde and acetate, the initial rate was similar to that with the blank, but was maintained. Ethanol gave a slightly greater rate, while butyrate, β-hydroxybutyrate, acetoacetate and propionate, with or without pantothenate, gave rates equal to those without added substrate.

Substrate competition. As the maintenance of constant rate of O_2-uptake was more important than an attempt to determine conditions for complete oxidation, the substrate concentration in these experiments was increased to 5 m*M* (Table 5). Under these conditions, although succinate promoted rapid O_2-uptake, the effect of pantothenate was relatively small, suggesting that the previous effect (Table 4) was due to oxidation of some of the products of succinate metabolism. The smaller effect of pantothenate was not due to inability of the suspension to take up O_2 at a greater rate, since succinate and pyruvate together gave a higher O_2-uptake in the

Table 5. *Effect of other substrates on oxygen uptake of deficient cells in presence of pyruvate and pantothenate*

Exp.	Substrate, 5 m*M* each optical component	Relative rate of O_2-uptake (rate with pyruvate and added pantothenate = 100): No pantothenate	With pantothenate	With pantothenate and pyruvate
1	Pyruvate	40	100	101*
	Succinate	121	133	176
	dl-Glutamate	17	49	106
2	Pyruvate	12	100	—
	Succinate	—	—	154
	α-Ketoglutarate	—	58	119
	Oxalacetate	—	109	105
	dl-Aspartate	—	35	117
3	Pyruvate	40	100	100*
	Glucose	167	187	188

* Double pyruvate concentration.

presence of pantothenate. The bulk of the O_2-uptake with succinate therefore did not go through the same system as that with pyruvate, but the small part increased by pantothenate may have done so. With glutamate, on the other hand, the maximum rate of O_2-uptake in the presence of pyruvate was little more than that with pyruvate alone, suggesting that a common system was involved. α-Ketoglutarate and aspartate behaved somewhat similarly to glutamate, while oxalacetate, as might be expected, showed almost the same uptake with or without pyruvate. No conclusion as to the role of pyruvate in glucose oxidation can be drawn, since the failure of pyruvate to increase the uptake in the presence of glucose can have no significance in the absence of evidence to show that substrate activation was the limiting factor in the chain of oxidation.

Influence of pantothenate on anaerobic metabolism

Of the nine substrates tested, all of which gave rapid O_2-uptake, only glucose, pyruvate and oxalacetate showed anaerobic fermentation detectable manometrically in $NaHCO_3$ buffer (Table 6). The fermentation of glucose, though more rapid than that of the other substrates, was of little interest from the point of view of the function of pantothenate, since it was little influenced by this substance. A slight decrease in the rate of gas production with glucose was observed in the presence of pantothenate (presumably due to diversion of pyruvate from the oxido-reduction with triosephosphate in the Embden-Meyerhof-Parnas scheme). The reaction was complete after the evolution of nearly 2 mol. gas calculated as CO_2. The rate was uninfluenced by excess fumarate except for the continued slow evolution of gas in the presence of pantothenate after the process without fumarate had ceased. The anaerobic metabolism of pyruvate, which was already known to be markedly dependent on pantothenate (Table 2) gave about 0·75 mol. gas (0·66–0·86 in 12 experiments) calculated as CO_2,

Table 6. *Effect of added pantothenate on anaerobic metabolism of deficient cells*

Relative production of acid + gas (calculated as CO_2) from various substrates in 1st hr.: pyruvate with added pantothenate = 100. In parentheses is given mol. gas produced/mol. substrate at the time of the completion of the reaction in the presence of pantothenate. Fumarate, where present, *ca.* 3700 μl., i.e. 50 m*M*.

Substrate	With substrate alone: No pantothenate	With substrate alone: With pantothenate	With substrate + fumarate: No pantothenate	With substrate + fumarate: With pantothenate	With substrate + pyruvate: No pantothenate	With substrate + pyruvate: With pantothenate
None	0	−8	8	8	15 (0·13)	100 (0·76)
Pyruvate	15 (0·13)	100 (0·76)	50 (0·44)	286 (0·95)	—	—
Glucose	900 (1·90)	820 (1·97)	935 (1·88)	910 (2·20)	—	—
dl-Glutamate	−18	−19	—	—	25 (0·11)	154 (0·76)
α-Ketoglutarate	0	6	19	36	73	145
Succinate	−2	−20	—	—	43 (0·20)	152 (0·74)
l(−) Malate	−3	−3	—	—	64 (0·29)	300 (0·81)
dl-Aspartate	3	1	−4	12	118 (0·75)	267 (0·84)
Oxalacetate	14	59	29	73	—	—
Glycerol	4	4	17	26	—	156

suggesting that two reactions already recognized in bacterial fermentation may be taking place:

(1) $2CH_3.CO.COO^- + H_2O = CH_3.CHOH.COO^- + CH_3.COO^- + CO_2$

as in the cocci [Krebs, 1937*a*] and *Esch. coli* at an acid *p*H [Krebs, 1937*b*];

(2) $CH_3.CO.COO^- + HCO_3^- = CH_3.COO^- + HCOO^- + CO_2$

as in aerobically grown *Esch. coli* [Krebs, 1937*b*] and in streptococci [Barron & Lyman, 1939].

In the presence of excess fumarate the rate was increased nearly threefold and the yield of gas became 1 mol./mol. pyruvate. This may be explained by assuming that fumarate replaced that part of the pyruvate acting as H-acceptor as in the oxido-reduction of reaction (1). Oxalacetate gave initial rates which were less than with pyruvate, and which fell off so that it was impossible to observe the completion of the reaction. The acceleration produced by fumarate in this case was also small. α-Ketoglutarate and glycerol stimulated no evolution of gas when present as sole substrates, but gave a small amount in the presence of fumarate. Glutamate, succinate, fumarate, malate and aspartate showed no evolution of gas, while aspartate did not produce gas even in the presence of fumarate. All these substances increased the rate of gas production in the presence of pyruvate by at least 50 %—in the case of the C_4-dicarboxylic acids (excepting succinate) by 100–200 %—without significantly altering the total amount of gas produced. Oxalacetate gave a similar increase in rate with pyruvate which was studied in more detail (Table 7). The reaction with 112 μl. pyruvate was complete in 2 hr. when 75 μl. (0·69 mol.) gas had been evolved in excess of the blank. With 224 μl. pyruvate the rate was little increased and the reaction was not quite complete in 4 hr., but with 112 μl. pyruvate and 112 μl. oxalacetate the reaction was complete in 1 hr., the initial rate having been doubled. The yield was 1 mol. gas/mol. pyruvate. Even in the absence of pantothenate this reaction was complete in 3 hr. Exp. 2 (Table 7) showed that 0·25 mol. oxalacetate was sufficient to double the rate of fermentation, while a small effect was shown with 0·05 mol. Exp. 3 (Table 7) showed that the effect was truly catalytic, since even if the blank values were deducted (and this is not always justifiable [Krebs, 1937*b*]), the increase in output of gas due to 22 μl. (0·10 mol.) oxalacetate was 26 and 37 μl. in 1 and 2 hr. respectively, while the oxalacetate alone produced less than half these amounts. Although oxalacetate increased the rate of fermentation of pyruvate even in the absence of added pantothenate, the effect was not so great, and was possibly due to residual pantothenate in the cells. In contrast, the effects of aneurin and oxalacetate on pyruvate dismutation by aneurin-deficient staphylococci were identical either singly or together [Smyth, 1940]. Smyth suggested that the function of aneurin was the carboxylation of pyruvate to form oxalacetate, the latter acting as a catalyst in the dismutation (cf. Krebs & Eggleston [1940] for pigeon liver). In the present work, Exp. 1 (Table 7) did suggest, in fact, that an equimolecular amount of oxalacetate excluded pyruvate as a H-acceptor (thus increasing the yield of gas to 1 mol./mol. pyruvate) and that this reaction was catalysed by pantothenate.

Table 7. *Catalysis of anaerobic metabolism in the presence of pyruvate by oxalacetate with or without added pantothenate*

	Substrates		μl. acid + gas produced in periods					
			0–1 hr.		0–2 hr.		0–3 hr.	
Exp.	Pyruvate μl.	Oxalacetate μl.	No panto-thenate	With panto-thenate	No panto-thenate	With panto-thenate	No panto-thenate	With panto-thenate
1	0	0	−11	−11	−11	−10	−7	−5
	112	0	1	44	12	65	19	72
	224	0	7	51	16	100	26	134
	0	112	16	11	27	22	33	26
	112	112	41	100	70	102	104	105
2	0	0	10	6	15	10	19	10
	224	0	15	34	26	66	35	99
	213	11	20	38	31	76	42	115
	168	56	25	60	42	108	57	145
3	0	0	9	2	14	6	20	12
	202	0	24	47	40	105	56	147
	0	22	13	12	18	15	23	21
	202	22	41	83	64	151	77	165

Aerobic and anaerobic metabolism of pyruvate and the influence of pantothenate

Optimum p*H*. Table 8 shows that the optimum *p*H for pyruvate oxidation, with or without pantothenate, was about 5·9, but the optimum effect of

Table 8. *Optimum pH for influence of pantothenate on O_2-uptake with pyruvate as substrate*

(Initial *p*H calculated from composition of Na phosphate buffers freshly prepared in CO_2-free water.)

	Q_{O_2} 30–75 min.	
Initial *p*H	No pantothenate	With pantothenate
ca. 4·6	31	39
5·9	37	66
6·6	28	61
7·0	20	58
7·3	16	47
7·8	11	40

pantothenate occurred at about *p*H 7. Above this *p*H the proportion of O_2-uptake due to added pantothenate was almost constant at 70 % of the total, suggesting that catalysis by pantothenate was being limited by the rate of formation of some intermediate, e.g. perhaps oxalacetate. Attempts to determine the optimum *p*H for fermentation by variation of the concentration of $NaHCO_3$ (Table 9)

Table 9. *Optimum pH for influence of pantothenate on anaerobic metabolism of pyruvate*

(Initial *p*H calculated from composition of $NaHCO_3$ buffer in equilibrium with 5·7% CO_2 in N_2.)

		μl. acid+gas			
		0–45 min. 1·5 m*M* pyruvate in *ca.* 150 m*M* salts		60–120 min. 3 m*M* pyruvate in *ca.* 280 m*M* salts	
$NaHCO_3$ m*M*	Initial *p*H	No pantothenate	With pantothenate	No pantothenate	With pantothenate
1	*ca.* 6·0	0	27	—	—
3	*ca.* 6·5	10	24	20	26
10	7·0	14	46	14	24
30	7·5	19	52	17	36
90	8·0	17	49	20	45
270	8·5	—	—	18	38

showed that it was not less than 7·5–8·0 at the salt concentrations normally used in this work. When the $NaHCO_3$ concentration was increased to 0·27 *M* to give *p*H 8·5 in equilibrium with 5·7 % CO_2 (NaCl being added to other vessels to maintain the same total salt concentration) the usual lag before fermentation reached its maximum rate was prolonged for at least ½ hr., but in the 2nd hour the optimum *p*H both for total fermentation and for the increase due to added pantothenate was 8·0. Under these conditions almost exactly 1 mol. gas was produced at the optimum, while at *p*H 8·5 little more than half as much was obtained, and at *p*H 7·0 little more than one-third. These figures must be accepted with caution, however, since cessation of fermentation was not shown to be due to exhaustion of substrate, and injury of the enzyme system at slightly unfavourable *p*H values by the high salt concentration was not excluded. At the lower salt concentration, 1 mol. gas was also obtained at *p*H 8·0 however, suggesting that under optimum conditions, reaction (2) occurred to the exclusion of the oxido-reduction (1).

Aerobic disappearance of pyruvate. Table 10 shows that without added pantothenate almost exactly 0·5 mol. O_2 was taken up during the period over

Table 10. *Relationship between pyruvate disappearance and O_2-uptake with and without added pantothenate*

(Strain 1707—grown on NH_3-cystine medium.)

	μl. reactant per vessel			
	No pantothenate		With pantothenate	
Time hr.	O_2-uptake	Pyruvate present	O_2-uptake	Pyruvate present
0	—	184	—	165
1	− 46	96	− 108	9
2	− 93	3	− 174	3
3	− 113	4	− 222	0

which the pyruvate disappeared, after which the O_2-uptake continued more slowly. With added pantothenate the rate of disappearance of pyruvate was at least doubled, the rate of O_2-uptake did not fall so much and had not fallen to zero when 1 mol. had been taken up. This experiment was carried out in phosphate buffer. With repetition of the experiment (Table 11) further information was obtained by determining the disappearance of acid

Table 11. *Relationship between pyruvate disappearance, R.Q. and acid disappearance, with and without added pantothenate*

(Bacteria from 300 ml. amino-acid medium in 5 vessels. 1·67 ml. suspending fluid per vessel. 20 min. at 37° to reach equilibrium with 5·7 % CO_2 in air before making observations.)

	μl. reactant per vessel				
	No pantothenate		10^{-5} *M* pantothenate		
	Initially	Change after 4 hr.	Initially	Change after 2 hr.	Change after 4 hr.
Pyruvate	481	−465	431	−418	−423
Bound CO_2	813	+ 75	832	+152	+216
Total CO_2	832	+572	879	+641	+769
O_2	—	−307	—	−397	−525
R.Q.	1·86		1·61		1·00

+ and − signs indicate appearance and disappearance respectively.

and formation of CO_2 by the method of Dickens & Šimer [1931] with $NaHCO_3$ as buffer. Without added pantothenate the average R.Q. over a 4 hr. period, during which all the pyruvate disappeared, approached the value 2·00, i.e. that for oxidation to acetate, though some further oxidation did occur.

With pantothenate the R.Q. was lower, even in the first 2 hr. (during which all the pyruvate disappeared), while in the second 2 hr. the data correspond to complete oxidation of some of the acetate presumably formed earlier. Unfortunately, the only evidence for the effect of pantothenate on pyruvate disappearance was that a smaller amount remained at the end of equilibration.

DISCUSSION

The evidence produced shows that the effect of pantothenate on metabolism was not confined to the oxidation of pyruvate to acetate, since a definite, though smaller, effect on O_2-uptake was seen after no measurable amount of pyruvate remained (Table 10), and possible degradation products of pyruvate such as acetate or formate themselves gave an effect on O_2-uptake (Table 4). Dorfman *et al.* [1942] concluded that the action of pantothenate may not be confined to that on pyruvate oxidation, though the bulk of their evidence was in favour of the idea that pantothenate acts on the conversion of pyruvate to acetate. It is a matter for future work to decide if the effect on O_2-uptake in the presence of acetate or formate is indirect. From this point of view the study of formate oxidation might be especially interesting since it promoted moderate O_2-uptake which was initially independent of pantothenate. In this connexion the experiments of Krebs [1937*b*] on the interaction of formate and fumarate in *Esch. coli* might be relevant. An indirect action, linked with the pyruvate system, did seem likely with the C_4 and C_5 dicarboxylic acids, on account of their effects both on O_2-uptake in the presence of pyruvate (Table 5) and on the fermentation of pyruvate (Table 6).

Whatever the role of pyruvate, however, in the oxidation of other substrates, quantitatively it was certainly the most important substrate influenced by pantothenate. Other vitamins are known to be concerned in pyruvate metabolism: (*a*) *Aneurin* as its pyrophosphate is concerned ultimately in pyruvate breakdown, though so far there is no agreement as to its precise function [cf. Barron, Lipton & Goldinger, 1941; Krebs & Eggleston, 1940; Lipmann, 1941; Quastel & Webley, 1941; Smyth, 1940]. (*b*) *Nicotinic acid* as diphosphopyridine nucleotide is concerned in the equilibrium between pyruvate and lactate, and also with that between oxalacetate and malate which, according to the views of Krebs & Eggleston [1940] and Smyth [1940], may be involved in pyruvate metabolism. (*c*) *Riboflavin* as riboflavin-adenine dinucleotide may be concerned in the later stages of H-transport, as in *Lactobacillus delbrückii* [Lipmann, 1939], though not directly concerned in substrate activation. Pantothenate should now be added to these as a component of pyruvate oxidase, though it has not been shown to play a part in the cell-free preparations from *Lactobacillus delbrückii* [Lipmann, 1939] and *Esch. coli* [Still, 1941]. Its participation, however, was not excluded since it was not shown that the preparations were free from pantothenate, and it is likely that the treatment was not drastic enough to release this factor from any protein with which it may have been bound. Pantothenate is known to be bound in liver, one of the richest natural sources, and is freed by autolysis [Williams, 1941; Williams, Truesdail, Weinstock, Rohrmann, Lyman & McBurney, 1938], but there was no autolytic process in the preparation of the enzyme of Lipmann or that of Still.

SUMMARY

1. In washed suspensions of *Proteus morganii* grown with suboptimal supplies of pantothenate, the addition of pantothenate increased the aerobic metabolism of pyruvate to a greater extent than that of 15 other substrates and the anaerobic metabolism of pyruvate more than that of nine other substrates tested.

2. The substrates which gave increases in O_2-uptake approaching that with pyruvate were six C_4 and C_5 dicarboxylic acids, and lactate. Little or no fermentation of these substrates, or of malate or glycerol, was detected manometrically, but all (except lactate, which was not tested) were shown to increase the rate of fermentation of pyruvate. With the C_4 dicarboxylic acids, including malate but not succinate, the increase was several-fold. With glutamate, α-ketoglutarate, aspartate or oxalacetate (and possibly succinate) as substrate, the increase in the O_2-uptake promoted by pantothenate tended to occur at the expense of that similarly promoted with pyruvate as substrate.

3. With glucose as substrate, fermentation was practically unaffected by pantothenate, and O_2-uptake was increased only in the later stages.

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Micro-method for Estimating Vitamin A by the Carr-Price Reaction

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In the micro-determination here described an attempt has been made to eliminate errors due to fading, differences in the colour of test and standard, and interference by non-specific absorption. The tube containing the reaction mixture is photographed in light of a selected wave-length range together with a series of standard tubes of equal dimensions (see Fig. 1), and the photographic film is analysed by means of photoelectric photometry. The advantages of this method are: (1) the working range is between 0·06 and 0·4 i.u. vitamin A in a sample; such small amounts cannot be determined by photoelectric colorimetry in the usual way, but it is necessary to work in this range when making serial investigations on the blood of small animals and is convenient in investigations on human beings; (2) rapid mixing is ensured as a consequence of the small volume of the reacting liquids (0·02 ml.); (3) a record of the colour produced can be obtained as early as 2 sec. after the initiation of the reaction, and its course can be followed photographically with accurate timing, thus avoiding the lag of galvanometer readings in photoelectric methods [Dann & Evelyn, 1938; Yudkin, 1941]; (4) the readings are independent of fluctuations in the light source during the reaction.

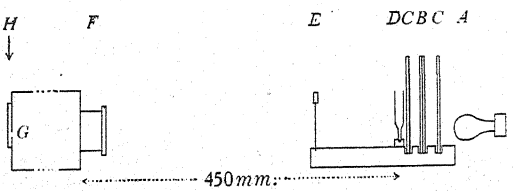

Fig. 1. Photographic arrangement. *A*, light source, two 100 W. coiled coil bulbs. *B*, space for filters: (1) 'Calorex' 2 mm.; (2) 'Signal green' 1 mm. (manuf. by Messrs Chance, 10 Princes St, S.W. 1). *C*, diffusing glass 2 mm. *D*, reaction tube and standard tubes. *E*, Wratten 26 red gelatin filter. *F*, enlarging lens at suitable distance to give image of tubes of about natural size. *G*, slit, 3·5 mm. wide, in front of slide which is moved in vertical direction. *H*, slide.

A detailed study of the error due to fading in the Carr-Price reaction is presented below. The principle of the method is generally applicable to measurement of colour in small volumes.

EXPERIMENTAL

Determination of vitamin A

Standards. Vitamin A. Any stable substance which has an absorption maximum in the region of 620 mμ is suitable as a standard. Solutions of cuprammonium sulphate in water were prepared ranging from 2·145 to 0·167 g. $CuSO_4.5H_2O$/100 ml., decreasing regularly by 20 %. The solutions contained 6 ml. conc. NH_3 (sp. gr. 0·88) and 15 g. $(NH_4)_2SO_4$/g. $CuSO_4.5H_2O$. The $(NH_4)_2SO_4$ stabilizes the colour in the presence of traces of alkali from the glass. Quantities of 0·04 ml. were measured into small glass tubes measuring 2 mm. inside diameter, 2·9 mm. external diameter and 30 mm. in length, and these were then sealed. The fused end was sufficiently flat to give a negligible error in the height of the column of fluid calculated from volumes exceeding 0·01 ml. The height was measured on a mirror scale and 0·3 mm. was added for the volume of the meniscus. The average diameter of 10 tubes cut from one piece of tubing varied less than ± 0·5 %; the variation was ± 1 % in two series of tubes. In order to eliminate differences in the diameters of individual tubes correction factors were applied to the concentrations. The standards were calibrated with solutions of vitamin A-naphthoate (2–22 i.u./ml. after addition of the reagent). Original solutions in arachis oil of 1000 i.u. vitamin A-naphthoate and of 300 μg. β-carotene/g. were supplied by British Drug Houses, Ltd.

β-Carotene. Standard tubes were prepared as described for the vitamin A standards and filled with solutions of $K_2Cr_2O_7$, containing 1 ml. conc. HCl (sp. gr. 1·18)/150 mg. $K_2Cr_2O_7$, in concentrations from 0·595 to 0·01865 g./100 ml. This standard matches well with solutions of carotene and extracts of human and rat blood and rat liver in light petroleum when compared visually in daylight without light filters through the range of 1·2–36 μg. β-carotene/ml. Acid bichromate neutralizes any alkali which may dissolve from the glass and its colour is nearer to that of carotene than bichromate. Calibration was carried out with solutions of pure β-carotene in light petroleum (B.P. 40–60°). Fig. 2 shows the calibration curve as obtained by visual comparison in daylight. The smallest amount estimated was 0·004 μg. carotene in 0·003 ml.

Procedure. The reaction tube was made by joining a piece of glass tubing of a diameter equal to that of the standards to a wider glass tube of 7 mm. inside diameter, and fusing the narrow tube at such a distance as to provide a length of about 15 mm. and in such a way as to give it a flattened end. The extract freed from water was pipetted into the reaction tube and the solvent evaporated in a stream of N_2 in a bath at 50–55°. The wall of the tube was repeatedly rinsed with small amounts of light petroleum (40–60°) and the latter evaporated off. After addition of 0·005–0·03 ml. of light petroleum the tube was stoppered and centrifuged. The volume of the solution was calculated from the height of the column, allowing 0·3 mm. for the meniscus. The β-carotene layer was then mixed with the light petroleum and the carotene estimated by comparison with the bichromate standards. For many purposes no greater accuracy than ± 5 % is required and comparison by the eye is sufficient. Greater accuracy could be obtained by photometry, as will later be described for vitamin A.

The light petroleum was then evaporated in a stream of N_2. With extracts of blood it was sometimes necessary to transfer this solution from the small amount of precipitate present into another reaction tube with as little light petroleum as possible (0·04 ml.). The residue was taken up three times with pure $CHCl_3$, evaporated to dryness to remove all light petroleum, and finally dissolved in 0·005–0·015 ml. $CHCl_3$. Acetic anhydride was added by capillary pipette to give a final concentration of 1–1·5 % and the tube kept stoppered until the $SbCl_3$ reagent was added. The acetic anhydride forming the bottom layer was then mixed with the solution and the reaction tube put in position as shown in Fig. 1. The $SbCl_3$ reagent (saturated solution in $CHCl_3$ containing 1·5 % ethanol) was added from a capillary pipette having a long and very fine point. The flow of reagent was controlled by means of a piece of rubber tubing attached to the pipette. The point was kept centrally and near the surface, and the addition occupied about ½ sec. The volume of the reagent added was equal to that of the original solution, and was measured on a mm. scale in the horizontal position, 0·01 ml. corresponding to 30 mm. The first exposure of 1½ sec. was made 2 sec. after the reagent was added. Subsequent exposures of 1 sec. were made by moving the slide rapidly downwards at intervals of 1 sec. with the camera shutter open. The reaction tube was then stoppered and the volume of the liquid determined.

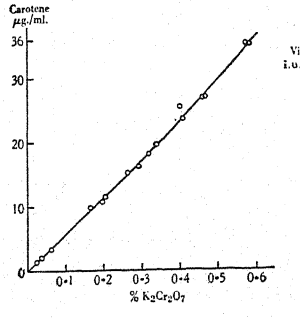

Fig. 2.

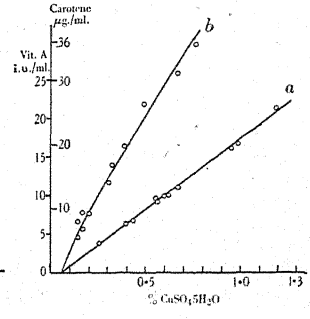

Fig. 3.

Fig. 2. Calibration curve for carotene.

Fig. 3. Calibration curve for the Carr-Price reaction of (*a*) vitamin A and (*b*) carotene.

The photographic films were analysed electrophotometrically under a microscope. A disk with a slit parallel to the photographic images to be compared was attached to the ocular mounting from which the ocular lens had been removed and a photoelectric cell of the barrier-layer

type was mounted at the upper end of the microscope. By moving the film under the microscope perpendicularly to the slit, galvanometer readings were obtained alternately for maximal transmission through the film (corresponding to maximal light absorption in a tube) and for the background of the film. The differences between the readings of the maxima and the background were plotted against the concentration of $CuSO_4$ in the standards, and the unknown value was read from the line connecting the values for the standards. This approximates to a straight line under the conditions used (Fig. 2). It is essential that the background be evenly illuminated. Photometry of three or four adjacent tubes in the range of the unknown concentration is sufficient. Fig. 3*a* shows the calibration line obtained with vitamin A-naphthoate. The arachis oil containing the vitamin A-naphthoate was diluted with light petroleum and aliquots were treated as described for extracts. The equation of the calibration line was found to be

$$y = 18{\cdot}25x - 1{\cdot}03,$$

x being the concentration of $CuSO_4.5H_2O$ as a percentage and y the concentration of vitamin A in i.u. The standard deviation was $\pm 3{\cdot}3\%$ in the range of 4–22 i.u./ml. With concentrations approaching 2 i.u./ml., corresponding to 0·04 i.u. in 0·02 ml. of reaction mixture, the error gradually rises to $\pm 15\%$.

The fading error in the Carr-Price reaction

It is generally accepted that the maximum colour of the Carr-Price reaction is proportional to the vitamin A present. Spectrographic examination confirms this view. Notevarp & Weedon [1936] showed that the blue colour obeys Beer's law. McFarlane & Sutherland [1938] achieved excellent agreement (average error 1 %) between the two methods in analyses of fish-liver oil concentrates, but the error was greater with the oils themselves (average error 6 %). Jensen & With [1939], analysing livers of mammals, birds and man, give the values found for $B^{1\%}_{1\,cm.}$ S 61/$E^{1\%}_{1\,cm.}$ 328 mμ as 2·70, 2·63 and 2·75 respectively, with $\sigma = 0{\cdot}404$, 0·436 and 0·406, where $B^{1\%}_{1\,cm.}$ S 61 represents the extinction coefficient of the Carr-Price reaction mixture (light filter S 61) and $E^{1\%}_{1\,cm.}$ 328 the extinction coefficient for light of wave length 328 mμ.

Variable fading rates have been observed by Dann & Evelyn [1938] not to affect the maximum colour intensity; and Meunier & Raoul [1938]

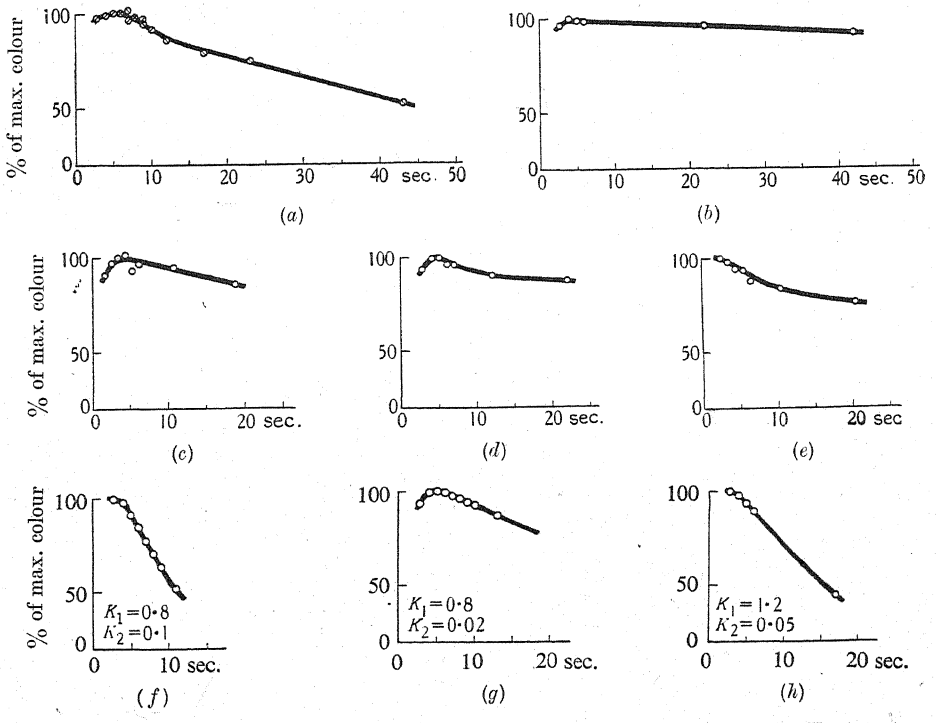

Fig. 4. Fading of Carr-Price colour given by different preparations with vitamin A activity. (*a*) Vitamin A-naphthoate: ⊘ 0·380 i.u. (16·7 i.u./ml.); ⊘ 0·283 i.u. (12·9 i.u./ml.). (*b*) β-Carotene: 0·77 μg. (35·5 μg./ml.). (*c*) Blood extract: 0·135 i.u. vitamin A (5·55 i.u./ml.); 0·143 μg. carotene (5·9 μg./ml.). (*d*) Blood extract with low concentration of vitamin A and high concentration of carotenoids: 0·054 i.u. vitamin A (2·9 i.u./ml.); 0·337 μg. carotene (16·7 μg./ml.). (*e*) Rat-liver extract: 0·264 i.u. vitamin A (13·0 i.u./ml.); 0·041 μg. carotene (2·0 μg./ml.). (*f*), (*g*) and (*h*): calculated fading curves.

suggest that they may serve as additional criteria for distinguishing vitamin A from vitamin A_2.

No information could be found concerning colour development during the first ½ min. after the start of the $SbCl_3$ reaction. This was studied by the method described above in samples of pure vitamin A-naphthoate and β-carotene, and in extracts of human blood and rat liver. If the maximum colour is reached before fading starts, these curves (Fig. 4) indicate a variable onset of the latter and variable rates of fading. If, however, fading begins as soon as the coloured compound is present, i.e. at the time of adding the reagent, the ideal maximum is never reached, but its value could be calculated by applying a correction, derived from the fading rate, to the observed maximum.

The calculations are based on a first order type reaction curve both for colour development and for fading in the neighbourhood of the maximum. The fading rate is assumed to be proportional to the concentrations of the molecules of vitamin A which had reacted with the $SbCl_3$, giving the blue colour. The relation between observed blue colour, expressed as percentage of the maximum colour assuming that no fading has taken place, and the time, is given by

$$\frac{100x}{a_0} = \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}),$$

the solution of the differential equation

$$dx/dt = a_0 k_1 e^{-k_1 t} - k_2 x;$$

where x = concentration of vitamin A as chromogenic compound, a_0 = concentration of vitamin A at the beginning of the reaction, k_1 = reaction constant of the colour development, k_2 = reaction constant of the fading reaction, t = time in sec.

The values of the ordinates corresponding to a series of finite exposures as used in these experiments (1½ and 1 sec.) were calculated from

$$\frac{100}{a_0}\int_{t_1}^{t_2} \frac{x\,dt}{t_2 - t_1} = \frac{1}{k_2 - k_1}\left(-\frac{1}{k_1}e^{-k_1 t} + \frac{1}{k_2}e^{-k_2 t}\right)_{t_1}^{t_2}.$$

Fig. 4 shows curves (f, g and h) calculated by means of this formula with varying values for k_1 and k_2, in which the ordinates represent the values for $100x/a_0$ expressed as percentage of the highest value for $100x/a_0$ obtained.

It is possible to estimate k_2 directly from the slopes of the experimental curves at their points of inflexion after the maximum, when colour formation has practically stopped. From this figure and the value of the ordinate obtained at the first reading, k_1 can be determined by the formula, and with it, theoretically, the time of the true maximum.

k_2 was $0{\cdot}018 \pm 0{\cdot}003$ for vitamin A-naphthoate and 0·005 for carotene. Wide variation from 0·003 to 0·1 was observed with extracts from various sources. The value of the first ordinate in 55 experiments ranged from 90 to 100 % of the maximum value found. The accuracy of the determination of k_1 was limited by the practical difficulty of ensuring complete mixing within 2 sec.

When k_1 is taken as 0·7 and k_2 as 0·01, the first ordinate works out at 88·5 % of the maximum. Where k_1 is 0·7, or greater, and k_2 is 0·01, or greater, the position of the maximum according to the above formula must be at or earlier than 6 sec. after reaction. The positions of all maxima were found to fulfil this condition.

In dilutions below 4 i.u. vitamin A/ml. the curves often deviate from the calculated, the fading constant being greater at the beginning than would correspond to the value found at 22 sec. after reaction. The effect was not detected with concentrations above 4 i.u./ml. or with solutions of vitamin A-naphthoate. It may be caused by interfering substances in the blood. Agreement is obtained between curves calculated with $k_1 = 0{\cdot}8$ and experimental curves for concentrations greater than 4 i.u./ml., but the validity of the formula derived needs further test on account of the uncertainty of k_1.

Table 1. *The estimation of vitamin A and carotenoids and the fading rate of the Carr-Price colour with two concentrations*

Subject	Vol. of reaction mixture ml.	Conc. in reaction mixture i.u. vit. A/ml.	Conc. in blood per 100 ml. serum or plasma: i.u. vit. A	Conc. in blood per 100 ml. serum or plasma: μg. carotene	Serum or plasma used ml.	% fading for vit. A at 22 sec. after reaction
I.V.	0·0199	2·1	51	57	0·081	45
	0·0213	3·6	49	55	0·156	33
M.	0·0184	1·24	25	32	0·09	—
	0·0195	2·59	28	31	0·18	—
B.	0·0177	2·67	53	64	0·09	—
	0·0189	4·50	47	73	0·18	—
A.B.	0·019	4·37	103	90	0·081	24·3
	0·0213	7·52	103	90	0·156	18·2
J.R.M.	0·0187	5·27	97	78	0·102	5·5
	0·0216	7·24	87	83	0·18	8
R.B.	0·021	2·92	76	97	0·081	43
	0·0195	6·3	78	95	0·156	32
D.M.	0·0187	6·52	135	87	0·09	7
	0·0186	12·7	131	89	0·18	—
J.R.M.	0·021	4·12	85	90	0·102	—
	0·0196	7·55	82	89	0·18	—
W.	0·0177	2·66	52	74	0·09	11·7
	0·021	3·64	43	78	0·18	14·5

The observation that the percentage fading measured by the value at 22 sec. in two different concentrations of an individual extract is nearly the same (Table 1), may be taken as evidence of a first order type of fading curve. Whether the mechanism of the fading reaction is unimolecular or whether it is bimolecular with a second constituent in excess was not investigated.

It is of practical interest to know whether corrections should be applied to the maximum colour value observed in samples that fade at different rates. Table 2 gives the calculated maximum values that described for vitamin A (Fig. 3*b*). Values for concentrations lower than 8 μg. carotene/ml. were too small to be measured accurately. For the purpose of correcting the Carr-Price values of extracts

Table 2. *Calculated corrections to maxima, reached with different values of k_1 and k_2*

k_2 ...	0·005			0·01			0·02			0·03			0·05			0·1		
k_1	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}
0·8	97·2	−6	5·1	94·5	−3·5	14·0	90·9	+0·5	27·5	87·6	+4	38·7	82·9	+10	57·2	73·4	+24·5	82·7
1·0	97·7	−5	5·9	95·3	−2·5	15·1	92·3	+0·5	28·9	89·4	+4	40·4	84·5	+10	58·5	76·6	+21·5	83·9

Max. = calculated maximum value of colour as percentage of theoretical total, without fading.
Corr. = corrections to experimental maximum values as percentage.
f_{22} = fading 22 sec. after reaction as percentage of the maximum.

as percentages of the theoretical values without fading (1st columns), and the corresponding corrections for samples whose constants of fading differ from those of vitamin A-naphthoate (2nd columns). The 3rd columns give the percentages of fading 22 sec. after reaction.

The distribution of the constants of fading in 33 blood extracts is given below. These figures were calculated from the values for the blue colour due to the vitamin A, and it was assumed, though not quite correctly, that the yellow colour of the extracts was entirely due to β-carotene. Possible differences in fading rate of the blue colour formed with β-carotene with various extracts were disregarded:

k_2	<0·005	0·005–0·01	0·01–0·02	0·02–0·03	0·03–0·04	>0·04
No. of samples	2	4	11	14	2	0

The corrections for the majority of extracts from blood are within ±5% (see Table 2), and so they need not be applied for practical purposes, but with fading rates greater than $k_2 = 0{\cdot}05$ the observed maxima yield values which are appreciably too low, and corrections are necessary.

The correction for β-carotene in the Carr-Price reaction

The blue colour given by carotene with the Carr-Price reagent was measured in the same way as containing carotene the calibration line was extrapolated graphically to reach the point where the calibration line for vitamin A crosses the abscissa, and this value, 0·056% $CuSO_4.5H_2O$, was subtracted from all figures of the calibration curve for carotene. The reason why the calibration line for vitamin A does not reach the zero point may be a smaller light transmission of the control compared with that of water.

SUMMARY

1. A photographic device for the measurement of the Carr-Price reaction is described.

2. The rates of fading with different extracts were studied, and the corrections which it is necessary to apply to the values obtained were determined.

3. If the concentration of vitamin A in the blood is calculated from the observed maximum colour without correction for fading the error will generally not be greater than 5%.

I wish to thank the authorities of the Haymeads Emergency Hospital, Bishop's Stortford, and of the London Hospital for the facilities which made this work possible and the British Drug Houses, Ltd. for preparations of vitamin A and carotene.

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The Effect of Prolonged Administration of Carotene in the Form of Vegetables on the Serum Carotene and Vitamin A Levels in Man

By H. HOCH (Freedom Research Scholar), *From the Haymeads Emergency Hospital, Bishop's Stortford*

(*Received 16 March 1943*)

Few systematic investigations of the effect of prolonged administration of carotene on the blood level of carotene and vitamin A have been recorded. Kauffmann & v. Drigalski [1933] found that with continued administration of carrot juice to human subjects a maximum concentration of serum carotene (0·27 mg./100 ml.) is soon reached. Vitamin A levels were not investigated.

Wendt [1935] confirmed the occurrence of maximum values for carotene in blood in spite of continued carotene intake. The serum vitamin A, which at first rose, fell again on continued administration of carotene.

Ralli, Pariente, Brandaleone & Davidson [1936] administered various doses of 0·3 % carotene in oil daily for 2–4 months to normal and to diabetic persons. With the higher doses the serum carotene rose to 0·403 and 0·680 mg./100 ml. in one normal and one diabetic subject respectively during the period of dosing, and then in the normal subject further to 0·486 mg./100 ml. during the following 7 days. There was clinical evidence of 'carotenaemia' in diabetic patients when the level had risen above 0·500 mg./100 ml. Carotenaemia was not noted in normal persons. The vitamin A levels were not investigated.

The present investigation deals with the effect of prolonged administration of different amounts of carotene in the form of vegetables on the serum carotene and vitamin A levels in healthy human subjects. The circumstance that practically all food containing preformed vitamin A is rationed favoured this experiment, since the vitamin A intake could vary only within fairly narrow limits.

EXPERIMENTAL

The serum level of carotenoids and vitamin A was established in three females and two males during periods of 14–87 days prior to the feeding period. Exp. 1 and 2 were started in March 1942 after several months of a diet comparatively poor in carotene and vitamin A. The other three experiments were done during the summer. The duration of the diets with a high carotene intake ranged from 14 to 77 days. Determinations of carotene and vitamin A in the food were not carried out, but as it seemed desirable to have figures which would reflect the dietary fluctuations in these substances, detailed records of the diets were made weekly and the total consumption of carotene and vitamin A was calculated from the data shown in Table 1. The rationed food was considered separately and the deviations from the basal spring ration (approximately 10,000 i.u./week) were plotted on the charts as fractions of the total. The higher values for the vitamin A intake are due to the greater allowances of milk and cheese during the summer. The uncertainty as to the figures for the carotene content of the vegetables is greatest with those which entered the records as 'greens'. It is indicated by the shaded areas in the diagrams, the assumption being made that the biologically available carotene content might vary between 3000 and 9000 i.u./100 g.

In Exp. 1, 2 and 4 serum was analysed which had been obtained by finger prick from fasting subjects in the morning. In Exp. 5 blood was drawn from a vein and the plasma was analysed. In

Table 1. *Relative vitamin A activity of foods*

(*a*) Vegetables

From 'The vitamin A value of carotene in vegetables' [Graves, 1942]

	i.u./100 g.
Spinach	12,000
Watercress	12,000
Broccoli leaves	12,000
Carrots	7,500
Lettuce	3,000
Tomatoes	3,000
Brussels sprouts	1,700
Peas	1,000
Cabbage	1,000
French beans	700

[Jeghers, 1937]

(*b*) Rationed foods

	i.u./week	
Butter, 2 oz.	3000	[Coward, 1938]
Margarine, 4 oz.	2000	
Milk, 3 pt.	3900	Calculated from the above figure for butter, assuming 86·85 % fat in butter [Winton & Winton, 1937] and 3·75 % fat in milk [Coward, 1938]
Cheese, 2 oz.	420	[Davies & Moore, 1939]
Egg, 1	400	[Cruikshank, 1941]

Exp. 3 and 5 the samples were taken 2–3 hr. after breakfast. Analyses were made once a week or oftener. Carotenoids and vitamin A were estimated colorimetrically as described by Hoch [1943]. The carotenoids were expressed as μg. β-carotene and the vitamin A as i.u./100 ml. serum or plasma. Carotene and xanthophyll were not determined separately, although in Exp. 1 and 2 considerable portions of the carotenoids in blood may have been xanthophyll, due to the ingestion of spinach.

Method of extraction. The method used by Skurnik & Suhonen [1939] for the extraction of liver was employed, with modifications which prevent the presence of a protein precipitate at the beginning of the extraction with light petroleum; a heavy precipitate may impede the extraction of carotenoids and vitamin A [Yudkin, 1941]. To 0·08 (or 0·157) ml. of serum, contained in a narrow centrifuge tube (8·5 × 95 mm.) were added first 0·012 (or 0·024) ml. 40% aqueous KOH, and then, gradually, absolute ethanol to a final concentration of 30%. The air in the tube was replaced by N_2 and the tube was stoppered with a cork, fixed tightly to the tube with a loop of string, which itself was held in its place on the tube by a piece of rubber tubing. The mixture was heated in boiling water for 5 min. The tube was centrifuged to remove condensed material from the edge of the stopper before being opened, about 2 ml. of light petroleum (B.P. 40–60°) were added and the tube was then shaken for 10 min. The light petroleum was transferred to another tube of the same size with a special pipette (Fig. 1). Pipettes of this shape proved useful for the transfer of volatile fluids, the spilling of fluid from the pipette by too strong suction being prevented by the double bend. To the alkaline serum-ethanol mixture in the first tube absolute ethanol was added to raise the concentration to 50%. The mixture was again heated, under N_2, for 1½ min. The tube was centrifuged and, after addition of about 2 ml. light petroleum, was shaken as before. The pooled extracts were washed three times with distilled H_2O, once with about 2*N* HCl* and again three times with H_2O. The layers were separated by centrifuging and the bottom layer was removed with the pipette which had been used for transferring the light petroleum. The extract was then dried over anhyd. Na_2SO_4, evaporated to dryness, taken up with light petroleum and analysed for carotenoids and vitamin A.

Fig. 1. Pipette for transferring volatile liquids.

* In a few cases, previous to the use of HCl at this stage, turbid reactions were obtained with the $SbCl_3$ reagent in spite of the presence of acetic anhydride. With the procedure described here, the reaction mixture was always optically clear. The absence of the Tyndall effect was ascertained in every case.

Fig. 2 and 3 illustrate the results obtained in Exp. 1 and 2. The average intake of vegetable sources of carotene was ½ lb. of carrots/day or its carotene equivalent in other vegetables, mainly spinach (see Table 1), for periods of 63 and 77 days.

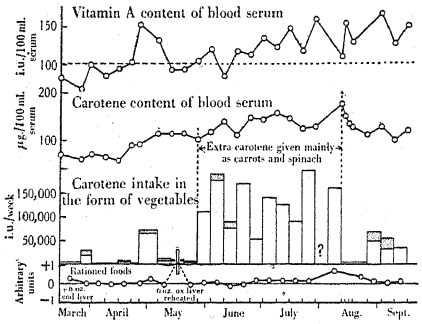

Fig. 2. Exp. 1. A.B., ♀, 24 years; ←——→ period of average weekly intake of about 120,000 i.u. of carotene, mainly as carrots and spinach.

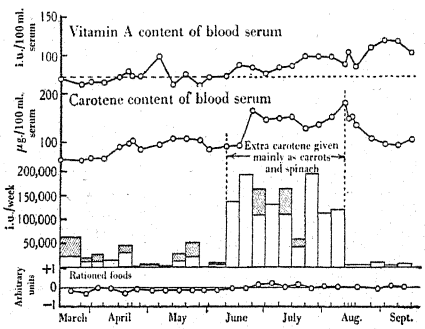

Fig. 3. Exp. 2. R.B., ♀, 24 years; ←——→ period of average weekly intake of about 120,000 i.u. of carotene, mainly as carrots and spinach.

The carotene level of the serum showed an increase from 60 to about 100 μg./100 ml. in the period before extra carotene was given, due to increased intake of vegetables in the free diet. It then increased further to about 150 μg. and this level was maintained during the period of carotene feeding with fluctuations parallel to the intake. On discontinuation of the surplus of vegetables the carotene level fell slightly (to 115 and 90 μg.) in Exp. 1 and 2. In Exp. 5 the plasma-carotene values varied between 120 and 160 μg. before and during the feeding period and between 150 and 180 μg. after the feeding period.

The serum vitamin A level started to rise, apparently within the first 2 weeks of the feeding period, in Exp. 1 and 2. The average value changed in Exp. 1 from 102 before, to 147 i.u./100 ml. after, the feeding period, and in Exp. 2 from 71 to 105 i.u./100 ml. The vitamin A level remained unaltered in Exp. 5 at about 150 i.u./100 ml.

A different reaction was observed in Exp. 3 and 4 (Fig. 4), in which the diet was supplemented with 1 lb. of carrots daily for 25 and 32 days respectively. The carrots were included in the meals and consumed together with about 3 % of margarine or butter. They were steamed or otherwise cooked, or (Exp. 4) grated, and steamed with the margarine for 30–40 min. The carotene level rose continuously till the 19th and 20th day to 375 and 470 μg./100 ml. At this time the rate of increase of the serum carotene

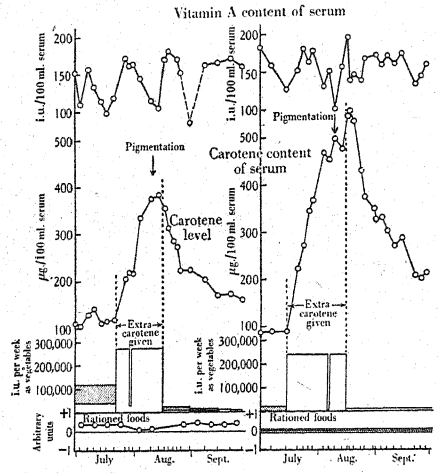

Fig. 4. Exp. 3. N.D.H., ♀, 29 years. Exp. 4. H.H., ♂, 33 years. ←——→ period of average weekly intake of about 240,000 i.u. of carotene as carrots.

slowed down considerably in spite of continued intake of carrots for further periods of 6 and 12 days respectively. The level remained high in case 4 for a further 4 days and it fell in both cases first at a rapid rate, then more slowly. The highest levels reached were 383 and 557 μg./100 ml. The vitamin A levels fluctuated around 143 and 155 i.u./100 ml. during the whole experiment.

At about the time at which the initial rate of increase in the level of carotene changed, the skin of both subjects became yellow, the pigmentation, particularly of the face and hands, being intense on the 20th and 26th day respectively. The sclera showed no discoloration. The pigmentation persisted with decreasing intensity for more than 16 days after discontinuation of the carrots in the diet.

DISCUSSION

The concentration of carotene in the serum must depend on the difference between its rate of uptake into the blood and its rate of elimination, which might include chemical destruction, excretion, and deposition in stores or in tissues where it is converted into vitamin A.

The statement that with a moderate intake of carotene the serum carotene fluctuates with the intake was confirmed in the present experiments. In two cases, however, in which there was a high intake of carrots (Fig. 2), some characteristic features of the carotene curves obtained allow the following interpretation.

The increase in serum carotene was very nearly linear during the first part of the feeding period. This steady increase makes it probable that the rate of uptake into the blood, as well as the rate of elimination, was constant. The time at which the bend of the curve occurs, after the ascending part, coincides almost exactly with the visible deposition of carotene in the skin. Assuming that this deposition, together perhaps with a deposition in other less common sites, started while the other mechanisms remained essentially unchanged, then its rate must be smaller than, or, at the most, equal to, the initial rate of increase. The total amounts thus calculated to be deposited daily in these new sites are very small compared with the amounts consumed.

The bend on the descending limb of the curve, after the almost linear fall for 10 and 8 days respectively, indicates an additional output of carotenoids from stores. The slow fading of the visible pigmentation, together with the abrupt fall of the serum carotene after the maximum, suggest a much retarded release of carotene from the skin and the stores.

It is remarkable that the levels of vitamin A in these two cases, and also in one case with medium carotene intake (Exp. 5), did not rise significantly above the levels (126, 155 and 150 i.u./100 ml.) which were found at the beginning of the experiments. A significant increase in the serum content of vitamin A on feeding medium doses of carotene was observed in two cases after a period of low vitamin A and carotene intake during the winter. The original levels were 102 and 71 i.u./100 ml., and the increase seemed to have started within the first 2 weeks. The intake before the experiment thus appears not to have been optimal. The vitamin A values were in all cases within the range considered as normal.

SUMMARY

1. The intake of carotene in the form of spinach and carrots in amounts corresponding to a biological activity of 120,000 i.u./week raised the serum

carotenoid content, expressed as β-carotene, from about 100 to 150 μg./100 ml. in two cases, and from 120 to 160 μg./100 ml. in a third case.

2. The vitamin A levels rose in two cases from 102 and 71 to 147 and 105 i.u./100 ml. respectively, and remained unchanged at about 150 i.u./100 ml. in the third case.

3. Consumption of amounts of carrots sufficient to supply about twice the biological activity provided in the first three cases, resulted in a rapid rise in the serum carotene content from 120 to 375 and 470 μg./100 ml. within 19 and 20 days respectively. Pigmentation of the skin appeared in both cases and it was intense on the 20th and 26th days.

4. The vitamin A levels did not change significantly. The single values fluctuated in the neighbourhood of 143 and 155 i.u./100 ml. during the whole experiment.

I wish to thank the authorities of the Haymeads Emergency Hospital, Bishop's Stortford, for the facilities which made this work possible.

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The Vitamin B_1 Content of National Flour and Bread—the Results of Comparative Tests by Various Methods*

A Report by

THE VITAMIN B_1 SUB-COMMITTEE OF THE ACCESSORY FOOD FACTORS COMMITTEE OF THE MEDICAL RESEARCH COUNCIL AND THE LISTER INSTITUTE

(*Received 20 April 1943*)

At the request of the Ministry of Health, an investigation has been made into the merits of the various methods available for the determination of vitamin B_1† in national flour, in order to arrive at the most suitable procedures for use in chemical laboratories where food analyses are regularly made. The present report includes a comparison of chemical with biological and microbiological assays. The vitamin B_1 content of bread made from one of the flours was also examined in order to determine the effect of baking.

The flours employed were authentic specimens of national flour, i.e. 85% extraction flour as defined in the Medical Research Council's second report on bread [Medical Research Council, 1941]. The bread was baked in a batch of 40 loaves of 2 lb. each by a usual commercial procedure. The loaves were sliced a few hours after baking and the slices placed on slatted shelves and dried at 37°. Drying was complete in 2 days when the bread, crust included, was powdered in a laboratory mill.

* The following laboratories and investigators took part in the tests organized by the Committee:

(A) F. W. F. Arnaud, County Analyst's Laboratory, Kent. (B) A. M. Copping, Lister Institute of Preventive Medicine. (C) H. E. Cox, Public Analyst. (D) E. R. Dawson, Distillers Co. Ltd., Research Laboratories. (E) L. J. Harris and Y. L. Wang, Dunn Nutritional Laboratory. (F) E. Hinks, Public Analyst. (G) E. B. Hughes, J. Lyons and Co. Ltd., Laboratories. (H) G. V. James, Roche Products Ltd., Analytical Laboratories. (I) D. W. Kent Jones, the Dover Laboratories. (J) S. K. Kon and S. Y. Thompson, National Institute for Research in Dairying. (K) T. Moran and R. G. Booth, Research Association of British Flour-Millers. (L) B. S. Platt and G. E. Glock, Nutrition Building, National Institute for Medical Research. (M) W. M. Seaber, Consulting and Agricultural Chemist. (N) W. Smith, Allen and Hanbury Ltd., Analytical Dept. (O) F. Wokes and J. G. Organ, 'Ovaltine' Research Laboratories.

† I.e. aneurin, known as thiamin in the United States of America; for the purpose of this report vitamin B_1 includes aneurin as it occurs in biological materials.

METHODS

Biological

Early work on which the biological methods are based is summarized in the Medical Research Council [1932] report: 'Vitamins: A Survey of Present Knowledge' (p. 149 for 'growth' and 'curative tests' and p. 137 for 'bradycardia' in rats). The rat-growth method (as employed by Copping) was that described by Chick & Roscoe [1929]. The rat-growth method (as employed by Harris), the rat bradycardia method and the 'cure of convulsions' method were described by Harris [1940–1].

Microbiological

The literature relating to the original observations which led up to the fermentation method is briefly reviewed in the Medical Research Council [1932] report quoted in the preceding paragraph (pp. 170–1). The method used in the present investigation is that of Schultz, Atkin & Frey [1942].

Chemical

Thiochrome method. The two procedures adopted by the Sub-Committee are given in full in Appendix A, with references to the literature.

Azo method. The method used is that described by Platt & Glock [1943] and is based on the formaldehyde azo method of Kinnersley & Peters [1927], and Prebluda & McCollum [1936].

RESULTS

The results obtained in the collaborative tests by the various methods employed are set out in Table 1. They show that all methods of estimation yield values which are in good agreement, especially for the flours.

Table 1. *Vitamin B_1 content of national flours and bread determined by various biological, microbiological and chemical methods*

(All values are given as μg./g. dry material and are averages of several estimations.)

Method	Flour C	Bread-crumb C	Flours: AGS 60	N.M. 116/42	N.M. 134/42	D	E	F	Investigator (see footnote, p. 433)
Biological:									
Rat growth: ♂	4·3	4·3	—	—	—	—	—	—	E
♀	4·4	4·9	—	—	—	—	—	—	E
(i)	3·1	3·3	—	—	—	—	—	—	B
(ii)	4·9	3·3	—	—	—	—	—	—	B
Cure of convulsions	3·2	3·0	—	—	—	—	—	—	E
Bradycardia	4·2	3·2	—	—	—	—	—	—	E
Average	4·0	3·7	—	—	—	—	—	—	
Microbiological:									
Fermentation	4·1	3·2	—	3·6	3·9	—	—	—	D
Chemical. Thiochrome:									
Visual comparison	3·5	—	—	—	—	—	—	—	C
	3·8	3·0	—	—	—	—	—	—	D
	4·2	3·4	—	4·3	4·2	4·2	3·9	4·2	E
	3·3	—	—	—	—	—	—	—	F
	4·0	3·2	3·0	3·9	3·8	3·6	3·4	—	G
	3·5	2·6	3·0	3·5	3·5	—	—	—	I
	3·6	3·2	—	—	—	—	—	—	M
Average	3·7	3·1	3·0	3·9	3·8	3·9	3·7	—	
Photoelectric comparison	3·9	2·9	3·0	3·4	3·5	—	—	—	D
	3·6	2·5	2·9	3·9	3·5	—	—	—	H
	3·5	2·8	2·9	3·4	3·5	—	—	—	I
	4·1	3·7	—	3·6	3·3	—	—	—	J
	3·9	3·0	3·0	3·6	3·6	3·7	3·1	3·8	K
	3·4	2·5	3·0	3·2	3·4	—	—	—	N
	3·7	3·2	3·0	3·3	3·3	—	—	—	O
Average	3·7	2·9	3·0	3·5	3·4	—	—	—	
Chemical. Azo	4·0	—	—	—	—	—	—	—	A
	3·5	—	—	—	—	—	—	—	C
	—	—	—	—	—	4·3	3·8	—	E
	3·6	—	—	—	—	—	—	—	F
	3·9	—	3·0	3·8	—	3·2	3·0	3·5	G
	4·4	—	—	—	—	2·8	3·1	3·5	L
Average	3·9	—	—	—	—	3·4	3·3	3·5	

The results of a statistical examination of the figures given in Table 1 are shown in Tables 2 and 3. In Table 2 are the values for vitamin B_1 for flour C and for breadcrumb C obtained from flour C, as determined by the biological and chemical tests.

Table 2. *Comparison of vitamin B_1 content ($\mu g./g.$) of flour C and breadcrumb C*

	Biological tests		Chemical tests	
	As received	Calculated on dry wt.	As received	Calculated on dry wt.
Flour C	3·9	4·4	3·4	3·9
Breadcrumb C	3·6	3·9	2·8	3·0

The average figures given were obtained by weighting the various results according to their accuracy.

The accuracy of these results has been determined and is set out in Table 3.

Table 3. *The limits of error of the results given in Table 2*

	Biological tests, %		Chemical tests, %	
	$P=0·95$	$P=0·99$	$P=0·95$	$P=0·99$
Flour C	76·4–130·9	70·2–142·5	±1·71	±2·27
Breadcrumb C	70·8–141·2	63·5–157·5	±4·18	±5·49

The difference between values of flour C and breadcrumb C is covered by the error of the test in the biological determination, therefore biologically they are apparently equal in value; but the difference in the chemical test is not covered by the error of the test, therefore chemically they are apparently of unequal value. In other words, there appeared to be a loss of vitamin B_1 on baking as judged by the chemical test, but the biological test was not accurate enough to confirm this.

Only four experiments, two or three observations in each by one observer, were made by the fermentation method, and data were insufficient to make any reliable comparison of the accuracy of this method with that of the chemical methods.

The limits of error obtainable by chemical methods in routine determinations of pure vitamin B_1 and of vitamin B_1 in national flours and bread are shown in Table 4. The calculation for pure vitamin B_1 by the thiochrome visual method was based on the results of a series of 24 determinations made by Dr Y. L. Wang on one solution of vitamin B_1. That for pure vitamin B_1 by the thiochrome photoelectric method was based on duplicate determinations on 100 different solutions made by Dr R. G. Booth. The calculation for the flours and bread is based on 61 observations (five observers) for the thiochrome method by visual comparison and 80 observations (seven observers) for the same method using photoelectric comparison. There were 22 observations (two observers) for the azo method.

It is doubtful if the photoelectric method is less accurate than the visual method; it is certainly not more so. It is also doubtful if the azo method is more accurate than the thiochrome methods, as fewer workers on the former sent in results on which to base an estimation of the accuracy of this method.

CONCLUSION

The thiochrome and azo methods as used in this work are considered to be reliable for the routine determination of vitamin B_1 in national flour.

(*Signed*) R. A. PETERS (*Chairman*)
H. CHICK
K. H. COWARD
L. J. HARRIS
B. S. PLATT
T. F. MACRAE (*Secretary*)

The Committee is indebted to the Research Association of British Flour-Millers for supplying and distributing specimens of national flour; to Messrs Matthew, Cambridge,

Table 4. *Limits of error obtainable in chemical determinations of pure vitamin B_1 and of vitamin B_1 in extracts obtained from national flours and bread*

	Thiochrome method, %				Azo method, %	
	Visual comparison		Photoelectric comparison			
	$P=0·95$	$P=0·99$	$P=0·95$	$P=0·99$	$P=0·95$	$P=0·99$
Pure vitamin B_1:*						
In duplicate	1·97	2·59	1·98	2·60	2·8	3·7
In triplicate	1·61	2·11	1·62	2·12	2·3	3·0
In quadruplicate	1·39	1·84	1·40	1·84	2·0	2·6
Flour or bread:†						
In duplicate	11·2	14·7	13·2	17·4	8·9	11·6
In triplicate	9·1	12·0	10·8	14·2	7·2	9·5
In quadruplicate	7·9	10·4	9·3	12·2	6·3	8·3

* The two sets of results obtained for the comparison of the thiochrome and the azo methods, are each the results of work done in only one laboratory. The limits of error by the azo method are for an amount of 30μg. Similar values have been obtained for amounts of 20–40μg.

† The errors for flours and bread include errors due to differences between laboratories.

for baking the bread; to Miss A. M. Copping for drying and distributing the bread; to Mr H. W. Kinnersley for submitting a memorandum on the technique of the thiochrome method; to Dr E. B. Hughes representing the Society of Public Analysts and Other Analytical Chemists; to Dr K. H. Coward and Dr J. C. Irwin for statistical analysis of the results; to the laboratories that carried out the trials and to all the observers who have made tests (see footnote, p. 433). During the absence of Dr Macrae on active service, Dr B. S. Platt acted as Secretary.

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Appendix A: Thiochrome Method for Estimating Vitamin B_1 in National Flour and Bread

The thiochrome test is based upon the work of Jansen [1936], who first adapted the oxidative formation of thiochrome to the determination of vitamin B_1. Two alternative techniques are here described, which have involved in their gradual evolution much labour and reduction of experimental difficulties; the one involves a visual and the other a photoelectric comparison of the formed thiochrome. That employing the visual comparison essentially follows the procedure of Harris & Wang [1941; see also Wang & Harris, 1942]; the photoelectric procedure is that of Booth [1940; 1942; see also Nicholls, Booth, Kent Jones & Ward, 1942]. Based upon the latter, there have been published already two reports on the vitamin B_1 control of national flour and bread by the Research Association of British Flour-Millers [1942] and the Ministry of Food [1942].

Jansen's work depended upon the previous findings: (*a*) by Peters that oxidation caused the production of blue fluorescent substances from vitamin B_1 [Peters, 1935]; (*b*) by Kuhn, Wagner-Jauregg, Klaverin & Vetter [1935] that a blue fluorescent sulphur-containing compound similar to dehydroaneurin could be isolated from yeast; this they called thiochrome; and (*c*) by Barger, Bergel & Todd [1935] that the latter (which they isolated and analysed) could be produced by the action of alkaline ferricyanide on aneurin. Its constitution was confirmed by synthesis by Todd, Bergel, Fraenkel-Conrat & Jacob [1936].

Jansen and his colleagues have used photoelectric methods for comparison of fluorescence; others use visual and still others such as Mukherji [1939] the Pulfrich photometer. Important steps have been the freeing of aneurin from biological combination by pepsin [Westenbrink & Goudsmit, 1938] and by takaphosphatase [Kinnersley & Peters, 1938] upon advice from Dr H. D. Kay. Valuable work upon the estimation of aneurin in foodstuffs was published by Pyke [1939] and by Houston, Kon & Thompson [1940]; see also Ritsert [1938]. An absorption step was used by Westenbrink & Jansen [1938] and by Hennessy & Cerecedo [1939]; for a further review see Hennessy [1941] and for a critique for fluorophotometers Loofbourow & Harris [1942].

Principle of method

The vitamin, after extraction and purification* by suitable methods, is oxidized to thiochrome, a blue fluorescent substance, which is estimated visually or by a fluorimeter. The methods for flour and bread described herein, which are based on practical experience and designed for speed and convenience of working, are not necessarily applicable to the estimation of vitamin B_1 in materials other than flour and bread.

Reagents. (Those marked [v] are required only for method employing visual comparison and those marked [p] only for method employing photoelectric comparison.)

Hydrochloric acid (A.R.): 0·02 *N* [v].
Hydrochloric acid (A.R.): 2% (w/v) [p].
Hydrochloric acid (A.R.): 10% (w/v) [v].
Sodium hydroxide (A.R.): 10% (w/v) [v].
Sodium hydroxide (A.R.): 20% (w/v) [v].
Sodium hydroxide (A.R.): 30% (w/v) [p].
Potassium ferricyanide (A.R.): 5% $K_3Fe(CN)_6$ (w/v).
Hydrogen peroxide solution: 20 vol. [v].
Sodium acetate-acetic acid buffer solution (*p*H 4·0): 36 parts of *M*/10 Na acetate (A.R.) and 164 parts of *M*/10 acetic acid (A.R.).
Bromocresol green solution: 0·04% [v].
Quinine sulphate (stock solution): 0·01 g. quinine sulphate (B.P.)/100 ml. 0·1 *N* H_2SO_4 [p].
Ethanol: 96% (redistilled till free from fluorescence).
Methanol: (redistilled till free from fluorescence) [v].
Isobutanol: (redistilled till free from fluorescence and shaken with water until saturated).
Toluene A.R.
Papain dry powder.
Takadiastase dry powder.

* A method of purification which completely obviates a 'blank' is one in which adsorbents are used; this method is recommended in U.S.A. and has received support in one laboratory in England.

Vitamin B_1 stock solution: 20 μg. vitamin B_1 hydrochloride (British National Standard for Vitamin B_1*) per ml. of 0·01 *N* HCl containing 25% (v/v) ethanol. For standardization, dilutions of this stock solution should be made with 0·01 *N* HCl on day of test.

General instructions

Identical laboratory conditions (especially in regard to temperature) should be secured for conversion of vitamin B_1 into thiochrome in both standard and unknown solutions. Test-tubes used in comparing the fluorescence of solutions by the visual method should be of non-fluorescent glass; the closed ends of the tubes should all be of the same shape; the tubes should be uniform in diameter, which should be such that 10 ml. occupy a column of 6–7 cm. For any one series of comparisons the diameters of the tubes should be identical.

Corks should be 'extracted' by refluxing for 1 hr. with ethanol, followed by 1 hr. with ether and finally by washing with isobutanol until the washings are non-fluorescent.

In order to avoid the introduction of fluorescent impurities, solutions should not come into contact with 'unextracted' corks, rubber (e.g. bungs or teats), or with tap greases containing blue fluorescent substances.

Only distilled water of high quality (preferably glass distilled) should be employed.

Solutions containing thiochrome should be kept away from bright light and comparisons of fluorescence made in a darkened room.

Method employing visual comparison of fluorescence

(1) *Extraction of the vitamin.* (*a*) Heat 2 g. of flour (or bread) with 15 ml. of 0·02 *N* HCl for 10 min. in a conical flask (50 ml.) on a boiling water bath. Stir the flask at intervals. Then add 5 ml. of Na acetate-acetic acid buffer (*p*H 4·0), mix and cool to approximately 40°. Using bromocresol green as external indicator check the *p*H; if necessary, adjust to *p*H 4·0 by the addition of either 10% HCl or 10% NaOH. To the pasty suspension add about 0·1 g. takadiastase and about 0·1 g. papain. Add a few drops of toluene as preservative and maintain at 40–45° for 16–18 hr. Cool to room temperature and add distilled water to make up to 25 ml. Centrifuge and retain the supernatant fluid. (The volume occupied by the undigested residue is small and is to be neglected.)

(*b*) For flour only, the following alternative procedure may be adopted. Make 2 g. flour into a cream with 25 ml. 2% HCl, allow to stand 16–18 hr. at room temperature, agitate and then centrifuge until the supernatant fluid obtained is only slightly cloudy.

(2) *Removal of fluorescent impurities by washing with isobutanol.* To 5 ml. of the supernatant fluid contained in a centrifuge tube (15 ml.) add a drop of 10% HCl, to ensure that the solution is more acid than *p*H 4·0, followed by 5 ml. of isobutanol. Stopper the tube with a cork, shake for not less than 1 min., centrifuge, remove the upper isobutanol layer, retaining the aqueous layer, the volume of which should be recorded.

(3) *Development of thiochrome from vitamin B_1 in the purified extract.* (*a*) Take three stoppered glass cylinders* (25 ml.) labelled A, B and C. Into A (the unknown) introduce 1·5 ml. of aqueous layer, add 2 ml. methanol, then 1 ml. 20% NaOH followed within 30 sec. by 0·15 ml. $K_3Fe(CN)_6$ solution, mixing thoroughly after each addition. Into B (the blank) measure 1·5 ml. aqueous layer, 2 ml. methanol and 1 ml. 20% NaOH. Into C (the control) put 1·5 ml. diluted standard vitamin B_1 solution containing 4 μg. of the vitamin followed by 2 ml. methanol, 1 ml. 20% NaOH and 0·15 ml. $K_3Fe(CN)_6$ solution, observing exactly the same procedure as for A.

To the mixture in cylinders A, B and C add 0·25 ml. H_2O_2, mix and allow to stand $\frac{1}{2}$–1 min. Add 10 ml. isobutanol to each of the three cylinders, shake vigorously for 2 min. and allow the layers to separate. The upper isobutanol layer contains the thiochrome.

(*b*) In each case, draw off and discard the bottom aqueous layer; wash the isobutanol layer by shaking with 3 ml. distilled water for 1 min. Allow the layers to separate and transfer the upper layer to a dry conical flask. Then clarify by adding 2 ml. of ethanol.

(4) *Comparison of the fluorescence.* Measure 10 ml. of the clear isobutanol extract from the 'unknown' (A) and the 'blank' (B) into separate test-tubes. Compare in a dark room in front of the Wood's glass window of an ultra-violet lamp. Add the standard 'control' isobutanol extract (C) in small portions from a graduated pipette or burette to the 'blank' extract (B) until the fluorescence observed on looking down the tube matches exactly that of the 'unknown' (A). After each addition mix by stoppering with a cork and inverting. The tubes should be reversed frequently during the matching to avoid errors due to uneven illumination. Prior to the final matching an amount of isobutanol equal in volume to that of the 'control' isobutanol extract (C) added to the 'blank' (B) should be added to the unknown (A). It is advisable to use a Wratten 18A or similar filter in conjunction with Wood's glass to reduce errors due to substances with fluorescences of tints other than the purplish blue tint of thiochrome. (The H_2O_2 solution should have greatly reduced any non-specific yellowish fluorescence.) Matching must be related to the purplish blue tinted fluorescence of thiochrome, any other tint being ignored. Matching must be carried out rapidly since prolonged illumination increases eye fatigue, and the risk of photochemical destruction of thiochrome. Best comparisons are obtained by exposure and observation of 2–3 sec. periods, followed by periods of rest.

(5) *Calculation of results.* If x is volume in ml. of 'control' isobutanol extract (C) required and r_1 and r_2 the volumes in ml. of the extracts respectively before and after preliminary washing with isobutanol (para. 2), then the aneurin content of sample is

$$\frac{4x}{10} \times \frac{25}{1{\cdot}5 \times 2} \times \frac{r_2}{r_1} = \frac{10xr_2}{3r_1} \mu\text{g./g.}$$

Method employing photoelectric comparison of fluorescence

For use in the Spekker or other suitable fluorimeters, higher initial concentrations of flour or bread extracts may

* Obtainable by application to the Director of Biological Standards, National Institute for Medical Research, N.W. 3.

* Other types of reaction vessels may be utilized, e.g. glass-stoppered 1 oz. bottles or small separating funnels if the reagents are mixed by shaking, and boiling tubes if mixing is achieved by bubbling compressed air or nitrogen from a cylinder.

be required. In such cases, owing to the large bulk of solid matter (extracted flour or bread) present, it is necessary to carry out comparisons of standard against both the plain flour or bread, and flour or bread with a suitable aneurin addition. This dual comparison serves two purposes: (*a*) it makes allowance for substances which may be present and which interfere with the amount of fluorescent light which falls on the photocell by absorbing either the incident activating light or the emitted fluorescent light—in other words it serves as a recovery test—and (*b*) it enables the aneurin content of the extract to be related quantitatively to that of the flour or bread.

(6) *Extraction of the vitamin.* (*a*) The following procedure applies in particular to the Spekker fluorimeter but may readily be adapted to other fluorimeters. Take two 10 g. lots of flour or bread (A and A_1) in 100 ml. flasks; to A add 50 ml. acetate buffer from a graduated cylinder or burette and to A_1 a known amount of aneurin contained in a small volume of liquid, followed by a sufficient amount of acetate buffer to make the total volume of the additions 50 ml. A convenient amount of aneurin to add is 40 μg. (2 ml. stock solution of standard), representing the addition of 4 μg. aneurin to each gram of flour or bread being extracted. The suspensions of flour or bread should be free from lumps. To each flask add about 0·1 g. takadiastase and about 0·1 g. papain and a few drops of toluene as preservative and maintain at 40° overnight. Agitate and centrifuge and retain the supernatant fluids.

(*b*) For flour only, the following alternative is satisfactory. Take the two 10 g. lots of flour (as in 6 (*a*)); to one add 50 ml. 2% HCl and to the other the aneurin addition (as in 6 (*a*)) and sufficient 2% HCl to bring the total volume of the additions up to 50 ml. Allow to stand overnight at room temperature, agitate, centrifuge, and retain the supernatant fluids.

(7) *Removal of fluorescent impurities by washing with isobutanol.* This step is only infrequently necessary when using the photoelectric method. When, by visual inspection in ultra-violet light, the final isobutanol solution of thiochrome is seen to have strong non-characteristic fluorescence (usually white) superimposed on the characteristic purplish blue of the thiochrome, this step becomes necessary. It may be carried out exactly as in para. 2. Owing to dilution of the aqueous extract during the process, a factor r_1/r_2 (generally 11/10, see para. 5) must be applied in the final calculation of results.

(8) *Development of thiochrome from vitamin B_1 in the extracts.* Take three 6 × 1 in. boiling tubes, or alternatively stoppered vessels, labelled A, B and C. Into A and B, pipette 2 ml. of the plain flour or bread extract and into C, 2 ml. of the flour or bread extract containing added aneurin. To each tube add 2 ml. of methanol.

Then to tube A (the blank) add 1 ml. 30% NaOH solution and to tubes B and C, 1 ml. 30% NaOH followed within 30 sec. by 0·25 ml. $K_3Fe(CN)_6$ solution. The contents of the tubes are either kept constantly agitated by bubbling a stream of air or N_2 through them during the course of all additions or are mixed by shaking after each addition, according to whether boiling tubes or stoppered vessels are employed. Oxidation is allowed to proceed for a minimum of 1 min., after which 25 ml. isobutanol are added and the bubbling is continued for 2 or 3 min., or the stoppered vessels are shaken vigorously for about 1 min.

The aqueous and isobutanol layers are allowed to separate for about 15 min. to reduce turbidity, 1 ml. ethanol is added to each tube and is mixed carefully into the upper (isobutanol) layer (by means of the stream of air or a glass rod). The isobutanol layer is then decanted into the fluorimeter cell. Slight turbidity of this solution has not been found to influence the fluorimeter results.

(9) *Comparison of the fluorescence.* When the Spekker fluorimeter is used the fluorescence of the unknowns should be measured in terms of a standard which is stronger than the unknowns. As thiochrome is rather unstable in ultra-violet light, it is unsuitable as a standard, and quinine sulphate has therefore been adopted for the purpose. A suitable strength of solution is 1 μg. quinine sulphate/ml. 0·1 N H_2SO_4, and this can be made up monthly from the stock solution (kept in the dark) of 100 times this strength. The 'standard' cell is, however, freshly filled with the quinine solution daily. The instrument is calibrated against the quinine standard by means of a series of aneurin standards (e.g. 0·1–3·0 μg. for the Spekker), which have been prepared by oxidizing standard solutions of aneurin by precisely the same technique as is employed for flour or bread extracts. A standard curve is plotted relating E to μg. of aneurin, and readings are taken with the three extracts prepared above. If it happens that the concentrations suggested are outside the limits of the instrument, the estimation should be repeated, using a more suitable amount of extract, since dilution of the thiochrome layer is not permissible.

(10) *Interpretation of results.* The indicated vitamin B_1 content of the flour or bread extract minus the 'blank' is not precisely the vitamin B_1 content of the aliquot of extract, owing to the possibility that interference may lower the fluorescence. This is allowed for (at the same time relating the flour or bread extract to the flour or bread itself) by the use of the flour (or bread) plus aneurin values. It is known that the difference between the two flours or breads is precisely 4 μg. aneurin/g. Then, to take a concrete example, if an aliquot of the plain flour minus blank showed 1·5 μg. and the flour plus aneurin minus blank showed 3·5 μg. per aliquot, it follows that the aneurin content of the flour is

$$\frac{4}{3{\cdot}5 - 1{\cdot}5} \times 1{\cdot}5 \text{ or } 3\,\mu\text{g./g.}$$

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An Azo Colour Reaction for the Determination of Vitamin B_1 in National Flour

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Jansen & Donath [1926 *a*, *b*; 1927] reported that vitamin B_1 concentrates gave a Pauly colour reaction with diazotized sulphanilic acid. Kinnersley & Peters [1927; 1928], Windaus, Tschesche, Ruhkopf, Laquer & Schultz [1931] and Van Veen [1932], however, considered that vitamin B_1 did not give a Pauly reaction as ordinarily carried out or as modified by Koessler & Hanke [1919]. By increasing the alkalinity of the reaction as carried out by Koessler & Hanke, Kinnersley & Peters [1927] found that vitamin B_1 gave a pink colour instead of a yellow one, but this was tinged with yellow and faded gradually. Later, Kinnersley & Peters [1934] found that the pink colour could be intensified and stabilized by the addition of formaldehyde but that the yellow colour still persisted. Barger, Bergel & Todd [1935] suggested that it is the free amino group which reacts with diazonium salts. Todd & Bergel [1936], however, consider the formaldehyde-azo colour reaction of Kinnersley & Peters depends on the presence of a β-hydroxy-ethyl group in position 5 and a hydrogen atom in position 2 of the thiazole nucleus.

Diazotized *p*-aminoacetophenone was used by Prebluda & McCollum [1936; 1937; 1939] as a qualitative test for vitamin B_1 and by Platt & Yang [1937] and Yang & Platt [1939] for the quantitative estimation of vitamin B_1 in biological material, the reaction being carried out in the presence of the clay on which they adsorbed the vitamin B_1. Willstaedt & Bárány [1938] and v. Euler [1940–1] employed diazotized 2:4:dichloroaniline.

Melnick & Field [1939] also used Prebluda and McCollum's reaction in the quantitative estimation of vitamin B_1. They carried out a preliminary adsorption of the vitamin on 'Decalso', eluted it with hot acid KCl solution and developed the colour in the presence of phenol, which, they state, makes the reaction more sensitive, has a protective action on the oxidation of the vitamin B_1 in alkaline solution and prevents the inhibition of colour reaction caused by various substances. Emmett, Peacock & Brown [1940] adopted Melnick & Field's procedure except that they adsorbed the vitamin B_1 on 'Superfiltrol' instead of on 'Decalso', and carried out the colour reaction in the presence of the adsorbate, on which the dye was quantitatively adsorbed and subsequently eluted with ethanol. Auerbach [1940] has simplified Prebluda & McCollum's reagents and applied the reaction to the quantitative estimation of vitamin B_1 in certain pharmaceutical preparations, omitting the use of any adsorbent.

Prebluda & McCollum [1939] tested the specificity of the *p*-aminoacetophenone reaction, using several pyrimidine and thiazole derivatives. Some of the thiazole derivatives gave purple-red compounds with the alkaline diazo reagent. The free thiazole portion of the molecule reacted much more slowly and less completely than vitamin B_1 itself. They concluded that the presence of the methylene bridge between the thiazole and pyrimidine portions of the molecule was necessary for complete reaction. Melnick & Field found that degradation products of vitamin B_1 did not react. This was true after sulphite cleavage, which yields intact pyrimidine and thiazole derivatives; after the action of alkali and heat, which results in the opening of the thiazole ring; after simple deamination, and after oxidation to the thiochrome stage.

Kirch & Bergeim [1942] have recently described a colour reaction for vitamin B_1 with diazotized ethyl *p*-aminobenzoate, the dye being subsequently dissolved in *iso*-amyl alcohol. They claim that preliminary adsorption is unnecessary under their con-

ditions for the determination of vitamin B_1 in urine, but they have not applied the reaction to any other biological material. Using pure vitamin B_1 solutions, we have found this colour reaction to be much less sensitive than with diazotized *p*-aminoacetophenone using the procedure described below. We have attempted to apply their reaction to flour extracts and to 'Superfiltrol' adsorbates of flour extracts. In both cases, the colour reaction was completely inhibited, a bright yellow colour being extracted by the solvent.

We have recently examined the procedure of Melnick & Field and find that although their method of adsorption and elution is fairly satisfactory, it is a tedious and complicated procedure. A more simple method has been devised which was finally adopted for the estimation of vitamin B_1 in national flour.

EXPERIMENTAL

Extraction of vitamin B_1

The vitamin has been extracted from flour with 0·5*N* HCl and, in order to be sure of complete extraction, it has been found advisable to stir up the flour suspension at intervals over a period of several hours and finally to leave the suspension to settle overnight. Since flour contains a negligible amount of phosphorylated vitamin B_1, preliminary enzymic digestion has been omitted.

It has been found that during extraction the whole of the vitamin B_1 passes into the liquid phase. Thus, in calculating the vitamin B_1 content of flour, it is permissible to take the volume of the extracting fluid as the volume of the flour extract, so that if, as in the following procedure, 25 g. flour are extracted with 100 ml. 0·5*N* HCl, the vitamin B_1 contained in 40 ml. of the filtered extract is equivalent to 10 g. original flour.

Adsorption of vitamin B_1 from the flour extract

Quantity of adsorbent. This has been kept as small as possible so as to minimize the amount of dye precipitated on it at a later stage in the process. One adsorption of flour extract (=10 g. flour) on 50 mg. adsorbent removed approximately 90% of the vitamin B_1, a second adsorption on a further 50 mg. removing all the remaining vitamin B_1 from solution. Two successive adsorptions on 50 mg. 'Superfiltrol' have consequently been employed.

p*H for adsorption.* Quantitative adsorption of the vitamin B_1 from flour extracts was obtained without any adjustment of *p*H.

Time and conditions for adsorption. Shaking for a total period of 1 hr. on a tumbler rotating at approximately 60 r.p.m. has been found adequate for complete adsorption of the vitamin B_1 from flour extracts.

Adjustment of the adsorbate prior to the colour development

p*H adjustment.* Prebluda & McCollum [1939] advocated adjusting the *p*H to 5·0–6·0 immediately before carrying out the reaction. Melnick & Field [1939] give experimental evidence for adjusting the *p*H of vitamin B_1 in alcoholic solution to 7·0, but in practice add alkali until their solution is blue to thymol blue. Emmett *et al.* [1940] adjusted the *p*H in the presence of alcohol to 7–8.

We found that solutions at *p*H from 2–9, adjusted in the presence of alcohol, gave erratic results. Consistent and reproducible results were, however, obtained from *p*H 4–7, if the *p*H adjustment was made immediately before the addition of the ethanol.

Concentration of ethanol. The presence of a high concentration of ethanol in the final stages of the reaction is advantageous as it helps to elute vitamin B_1 from the adsorbent, stabilizes it, intensifies the colour produced [Melnick & Field, 1939] and also keeps the dye in solution. The concentration of ethanol (40%) cannot be exceeded, otherwise salts from the alkaline diazo reagent are precipitated.

Factors affecting the colour reaction

Preparation of alkaline diazo reagent. An alkaline reagent similar to that used by Kinnersley & Peters [1927] was incorporated in the reagents of Prebluda & McCollum [1939] and was used unmodified by Melnick & Field [1939], except that the latter workers carried out the colour reaction in the presence of phenol and ethanol. Prebluda & McCollum allowed the alkali and diazo reagent to react for at least 15 min. before carrying out the colour reaction. This procedure, we have found, diminishes considerably the colour produced.

Like Koessler & Hanke, we have found it necessary to add the alkaline diazo reagent to the alcoholic suspension of vitamin B_1 exactly 1 min. after mixing the diazo reagent and alkali.

The relative concentrations of alkali and diazo reagent have been found to be very critical. Employing the same reagents as Prebluda & McCollum, we have ascertained the effect on the colour development of varying the relative concentrations of diazo reagent and alkaline reagent. The two curves in Fig. 1 show the effects of (*a*) varying the amount of alkaline reagent from 1·0 to 5·0 ml., keeping the volume of diazo reagent constant at 2·5 ml., and (*b*) varying the amount of diazo reagent from 1·0 to 5·0 ml., keeping the volume of alkaline reagent constant at 2·5 ml. In both cases the reaction was carried out in the presence of a flour adsorbate, treated as described below, the colorimeter readings/100 mg. wet adsorbent being plotted against the volume of reagent (Fig. 1).

From these results it was concluded that for estimating the vitamin B_1 contained in 10 g. national flour, 2·5 ml. diazo reagent to 2·25 ml. alkaline reagent are required.

In the following method, the concentrations of acid *p*-aminoacetophenone solution and the alkaline reagent have for convenience been adjusted so that 2 ml. of both diazo reagent and alkali contain the optimal amounts of the reactants. The final diazo mixture is considerably less alkaline than that used by Prebluda & McCollum.

Presence of phenol. According to Melnick & Field, phenol intensifies the colour produced. Using our reagents we have, however, obtained much better colour development than did Melnick & Field employing phenol in addition. We consider the presence of phenol unnecessary.

Reaction time. Melnick & Field state that it is necessary to leave the reaction mixture overnight for complete colour development. Using the procedure described below, the maximum is reached at the end of 1 hr.

Extraction of the dye. Toluene is a more efficient solvent for the dye than xylene (used by Melnick & Field [1939]) in the presence of the adsorbent. The colour in toluene is, moreover, much more stable in bright daylight. Toluene, however, volatilizes more rapidly than xylene and prefrom the flour adsorbate reaction mixture alone with that from a flour extract to which vitamin B_1 had been added immediately before the colour reaction was carried out. The tints of the standard and unknown were identical by this procedure.

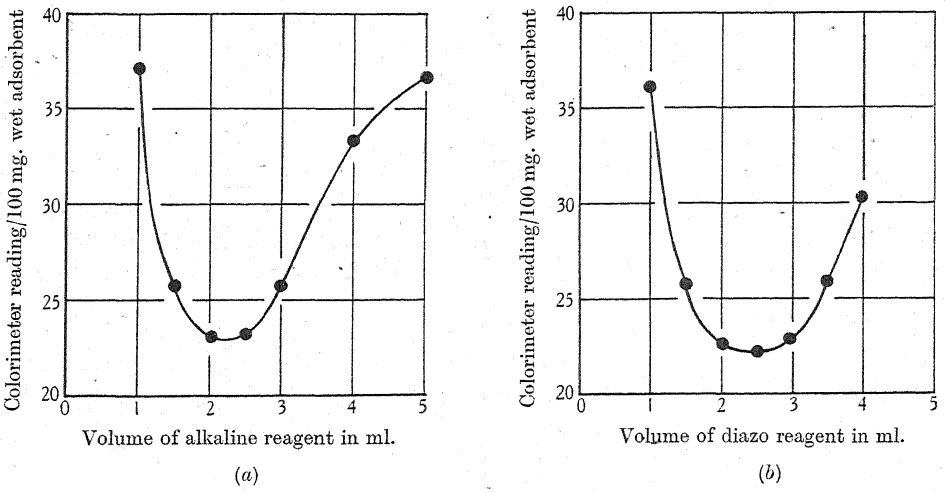

Fig. 1. The colour developed when flour adsorbate reacts with the diazo reagent in alkaline solution under different conditions: (*a*) with constant vol. (2·5 ml.) of diazo reagent, (*b*) with constant vol. (2·5 ml.) of alkaline reagent.

cautions have to be taken to prevent evaporation at any stage.

Emmett *et al.* [1940] adsorbed the dye quantitatively on 'Superfiltrol' and subsequently eluted it with ethanol. In order to simplify and shorten the procedure, we have attempted to keep most of the dye in solution by using a high concentration of ethanol, and have extracted the dye directly from solution by toluene. Extraction of the dye from the reaction mixture was found to be much more difficult if delayed until maximum colour development is reached, since, by that time, most of the dye is carried down on the adsorbent. Toluene was therefore added 20 min. after the beginning of the reaction. This procedure also accelerated the attainment of full colour development, which is never reached if extraction of the dye is begun during the initial 10 min.

Choice of standard

In early tests, the dye derived from flour extracts was compared with that from a standard vitamin B_1 solution, the colour reaction being carried out in the presence of 'Superfiltrol'. In the absence of 'Superfiltrol', the colour extracted from the vitamin B_1 reaction mixture was more purple than that extracted from a flour reaction mixture.

Values of more than 100 % for the recovery of vitamin B_1 were obtained when the vitamin was added to flour extracts before adsorption. Similar values were obtained if vitamin B_1 in solution was added after adsorption and immediately before the colour reaction was carried out. In both cases, i.e. with 20 μg. vitamin B_1 added before and after adsorption, a value of 22·6 μg. vitamin B_1 was obtained. It thus appears that the presence of the flour adsorbate intensifies the colour produced, and it was therefore decided to compare the colour of the dye extracted

Method adopted

Reagents

(1) 0·5*N* HCl (approx.).

(2) 2 % NaOH (w/v).

(3) Alkaline reagent: 3 % $NaHCO_3$ (w/v) in 0·6*N* NaOH.

(4) *p*-Aminoacetophenone solution: 0·8 % *p*-aminoacetophenone in 3·5 HCl (w/w). Keep in a dark bottle in the cold.

(5) 4·4 % $NaNO_2$ (w/v) (Analar). Keep in the cold, for not longer than 1 month.

(6) Diazo reagent: Measure 1·5 ml. *p*-aminoacetophenone solution into a 50 ml. volumetric flask and cool to 0–5°. Add 1·5 ml. $NaNO_2$ solution (previously cooled to 0–5°), mix and leave in the cold for 10 min. Add a further 6 ml. $NaNO_2$ solution, mix and leave for 10 min. Make up to 50 ml. with ice-cold distilled water and allow to stand at least 15 min. before use. (This reagent should be prepared on the day of the test and must be kept at 0–5°.)

(7) 'Superfiltrol' (activated clay).

(8) Toluene (rectified).

(9) 60 % C_2H_5OH (v/v) in distilled water.

(10) Standard vitamin B_1 solution: (*a*) Stock solution containing 150 μg. vitamin B_1 (hydrated—4·65 % moisture)/ml. in 0·01*N* HCl containing 25 % ethanol. (*b*) Solution for use: Dilute the stock solution with distilled water on the day of the test so that it contains 30 μg. vitamin B_1/ml.

Extraction of vitamin B_1 from flour and treatment of the adsorbate. (i) Stir 25 g. national flour into 100 ml. 0·5*N* HCl in a beaker, and leave at room temperature throughout the day preceding the test. Stir thoroughly at intervals and leave to stand overnight. Pour off the supernatant fluid and filter through a No. 1 Whatman filter paper (a satisfactory extract cannot be obtained by centrifuging).

(ii) Pipette 40 ml. of the filtrate into a bottle of 100–150 ml. capacity, provided with a glass stopper. Add 50 mg. activated clay, stopper and shake for 30 min. (a tumbler rotating at 60 r.p.m. is satisfactory). Transfer the contents of the bottle to a tapering 50 ml. centrifuge tube, wash out of the bottle as much of the adsorbate as possible with 5 ml. 0·5*N* HCl and add the washings to the contents of the centrifuge tube. Centrifuge until the adsorbate is well packed. Pour the supernatant fluid back into the bottle and shake for a further 30 min. with another 50 mg. activated clay. Transfer the contents to the centrifuge tube containing the first adsorbate and centrifuge. Discard the supernatant fluid. Wash out the bottle with five successive portions of 5 ml. 0·5*N* HCl, transfer the washings to the centrifuge tube containing the adsorbate and resuspend the adsorbate in the washings. Centrifuge again and discard the supernatant fluid.

(iii) Add to the washed adsorbate 1 ml. distilled water, 2 drops bromo-cresol-green and adjust to *p*H 5 (approx.) by the dropwise addition of 2% NaOH, keeping the adsorbate in suspension by stirring with a glass rod. Pour the suspension through a small glass funnel into a 25 ml. glass-stoppered measuring cylinder. Wash out the centrifuge tube with five successive portions of 2 ml. 60% ethanol, adding the washings to the suspension in the cylinder. Proceed immediately with the following step.

Development of colour. To 2 ml. NaOH-$NaHCO_3$ solution contained in a dry test-tube add 2 ml. diazo reagent. Invert to mix and, exactly 1 min. after mixing, add to the suspension of adsorbate in the measuring cylinder. Stopper and mix by inversion at intervals of a few minutes. After 20 min. introduce 5 ml. toluene into the cylinder. Restopper and continue to mix by inversion (done gently so as to avoid emulsion formation) at frequent intervals for a total period of 1 hr. or longer. Withdraw the toluene layer into another stoppered cylinder containing 10 ml. 0·5*N* HCl and wash by inversion. Transfer the washed toluene layer to a 5 ml. tapering centrifuge tube, shake in a small amount of anhydrous Na_2SO_4, mix and close the tube with a well-fitting cork. Centrifuge for a very short time, avoiding any risk of loss of solvent by evaporation.

Preparation of standard solution. To a second washed adsorbate, prepared as described in (i) and (ii), add 1 ml. of diluted standard vitamin B_1 solution and 2 drops of indicator and continue from this stage exactly as before with one difference, i.e. extract the dye with 10 ml. toluene instead of 5 ml.

Comparison of solutions. The two solutions are compared in a Duboscq type of colorimeter fitted with cups having a stem capacity of 5 ml.

Accuracy of method

(*a*) *Solutions of pure vitamin B_1.* The accuracy was determined on amounts of 20, 30 and 40 μg. pure vitamin B_1 in solution. The procedure adopted was the same as that described above for flour extracts except that preliminary adsorption on 'Superfiltrol' was omitted and it was found necessary to extract the dye with toluene after 60 min. instead of after 20 min., since the colour production is retarded in the absence of 'Superfiltrol'. The accuracy of the estimations (in triplicate) carried out by three different observers is set out in Table 1.

Table 1. *Statistical analysis of the results of vitamin B_1 determinations*

Amount of pure vitamin B_1 in test sample (μg.)	20		30		40	
Average of 9 observations (μg.)	20·11		30·12		39·98	
Standard deviation ...	0·4106		0·6099		1·0208	
Value of *P*	0·95	0·99	0·95	0·99	0·95	0·99
Limits of error:						
For determinations in duplicate	2·8	3·7	2·8	3·7	3·5	4·7
For determinations in triplicate	2·3	3·0	2·3	3·0	2·9	3·8
For determinations in quadruplicate	2·0	2·6	2·0	2·6	2·5	3·3

(*b*) *Extracts of national flour.* A number of results for the vitamin B_1 contents of national flours, using our azo method as described in this paper, obtained by different observers, have been published in a Medical Research Council report [1943]. The accuracy of the results obtained compares favourably with that of the thiochrome method.

SUMMARY

A simple colorimetric method is described for the routine estimation of vitamin B_1 in national flour using diazotized *p*-amino-acetophenone. The method, with minor modifications, can be used for the estimation of pure vitamin B_1.

We are indebted to Dr Katharine Coward for her statistical analysis of the results set out in Table 1.

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A Quantitative Method for the Determination of Uroporphyrin in Pathological Urines

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The chief difficulty attending the isolation of porphyrins from urine and their separation from one another is the relatively sparing solubility of these pigments in organic solvents. Coproporphyrins I and III are extractable by the acetic acid-ether technique discovered by Saillet [1896] and adopted by Fischer [1926] and may thus be quantitatively determined. For preparative purposes, although large volumes of solvent are required, a high degree of purification may be achieved by repeated transferences between ether and dilute HCl. Uroporphyrin III is similarly extractable by ethyl acetate from solutions adjusted to a *p*H of 3·0–3·2 [Waldenström, Fink & Hoerburger, 1935], but again large volumes of solvent are required for this laborious process, which is not suitable for quantitative purposes. Uroporphyrin I is not extracted by any known solvent immiscible with water.

Recourse must, therefore, be had to adsorptive procedures for the concentration and purification of the uroporphyrins prior to their spectrophotometric or fluorimetric determination. Many different materials have been proposed as suitable adsorbents for porphyrins. They include Ca and Pb phosphates, alumina, talc, $BaSO_4$, etc., and Waldenström [1935] has elaborated a chromatographic procedure for the adsorption of porphyrins from urine upon alumina. In this, as in all other procedures, adsorption takes place at or near the isoelectric point of the porphyrin. At this reaction the greater part of the dark brown or red water-soluble pigment of unknown constitution, always present in porphyrinuric urines, is also adsorbed, so that from a quantitative point of view the operation is of little value, and further purification of the uroporphyrin fraction is difficult. Attempted purification of the porphyrins from urine by acidifying with acetic acid [Nebelthau, 1899] is not always successful, and in any precipitate which forms coproporphyrin is mixed with uroporphyrin.

While searching for a new adsorptive technique, it was discovered that kieselguhr has the very desirable and remarkable property of adsorbing both coproporphyrins and uroporphyrins from solution *in dilute HCl* whilst rejecting almost entirely the dark, brown-red, non-porphyrin pigment. Elution by suitable buffers brings about a fairly sharp separation of the coproporphyrins from the uroporphyrins, but it has not been found possible to separate by this means the I and III isomers of either pigment, no doubt owing to the fact that their isoelectric points are so similar. Based upon the kieselguhr technique, a good quantitative method has been worked out for the determination of total porphyrin, and hence of uroporphyrin, in porphyrinuric urines, thus for the first time enabling

conditions such as acute idiopathic porphyrinuria to be subjected to accurate quantitative examination.

METHODS

Separation of pure coproporphyrin from uroporphyrin

The pigments used had been prepared from natural sources and purified by recrystallization of their methyl esters. These were saponified and the free porphyrins brought into solution in 5% HCl (w/w). Tswett columns were prepared from acid-washed kieselguhr (B.D.H.) suspended in 5% HCl (w/w) and the behaviour of the pigments studied individually on filtering their solutions through the columns, and on developing with buffer solutions. All porphyrins tested were strongly adsorbed from the acid solutions. Table 1 summarizes the results obtained with the developers. A mixture of uroporphyrin III and coproporphyrin I was adsorbed on a column from acid solution as previously described and then washed thoroughly with water. Acetate buffer of *p*H 5·25 was then filtered through the column, when the uroporphyrin passed readily downwards, leaving a sharply defined zone containing the coproporphyrin at the top of the column. The eluate contained no ether-soluble pigment. The coproporphyrin was finally removed from the column by a phosphate buffer of *p*H 7·15.

Table 1. *Behaviour of coproporphyrins and uroporphyrins adsorbed on kieselguhr*

Developer	Effect on porphyrin: Uroporphyrins I or III	Effect on porphyrin: Coproporphyrins I or III
20% HCl	Nil	Nil
50% acetic acid	Nil	Nil
Glacial acetic acid	Slow broadening of band	Slow broadening of band
Water	Nil	Nil
M Citrate buffer *p*H 3·2	Nil	Nil
M Acetate buffer *p*H 4·0	Nil	Nil
M Acetate buffer *p*H 4·5	Nil	Nil
M Acetate buffer *p*H 4·7	Broadening of band	Nil
M Acetate buffer *p*H 5·0	Broad ring passing slowly downwards	Nil
M Acetate buffer *p*H 5·25	All porphyrin removed	Very slow broadening of band
M Acetate buffer *p*H 5·5	—	Rapid broadening
M Phosphate buffer *p*H 6·5	—	Rapid broadening
M Phosphate buffer *p*H 7·15	—	All porphyrin removed

*p*H's measured by glass electrode.

Chromatographic separation of uroporphyrin from urine of acute porphyria

The specimen of urine, which was nearly black in colour, was passed during a period of severe attack by a man suffering from acute idiopathic porphyria. HCl was added to a concentration of 1% (w/w) and the mixture filtered through a column of kieselguhr. The deeply coloured filtrate was free from porphyrin. The column was washed well with 1% HCl (w/w) and then with glacial acetic acid, which removed the remaining dark brown pigment together with a little porphyrin detectable by fluorescence in ultra-violet light. After thorough washing with water, the uroporphyrin was then removed by acetate buffer of *p*H 5·25 and obtained as a deep red solution substantially free from other pigments. Upon adjustment of the *p*H to 3·0, the porphyrin precipitated readily, leaving a pale straw-coloured supernatant liquor. The precipitate was collected on the centrifuge, dried and esterified in the usual way. The ester crystallized from hot chloroform-methanol in fine microscopic needles, M.P. 258°.

The adsorption technique has also been used to purify the crude precipitate of mixed porphyrins obtained from a porphyrinuric urine by acidification with acetic acid. The mass was dissolved in dilute alkali and the *p*H adjusted to 5·25. Kieselguhr was then added and the mixture shaken vigorously and centrifuged. The adsorbent retained coproporphyrin, but little, if any, uroporphyrin. It is doubtful whether the method would be so effective were the proportion of coproporphyrin to uroporphyrin not so small (in the above case it was approximately 1:20), but by repeated shaking or filtration through a column it should be possible to remove substantially all coproporphyrin from uroporphyrin solutions by this simple expedient.

A quantitative method for the determination of uroporphyrin

The property possessed by kieselguhr of adsorbing uroporphyrin strongly from dilute acid solutions but of retaining very little of the accompanying urinary pigments provides the basis of a relatively simple method for the determination of uroporphyrin. The studies to be detailed below were carried out upon some specimens of urine from a case of acute idiopathic porphyria, and for his kindness in putting this material at my disposal I wish to thank Dr G. Discombe of St Bartholomew's Hospital.

Preliminary treatment of the urine. Waldenström & Vahlquist [1939] have shown that in acute porphyria a pigment precursor, porphobilinogen, is excreted, which is rapidly converted into porphyrin when the urine is acidified and warmed on the water bath [cf. Willenbücher, 1942]. During this heating, however, the urine becomes very dark owing to the formation of other, non-porphyrin, pigments and it has been found that the same full production of porphyrin is achieved without such undesirable accompaniment by rendering the specimen acid to a concentration of 1% HCl (w/w) and leaving it at room temperature overnight.

A known volume (usually 1–2 ml.) of urine containing approximately 5–50 μg. of porphyrin is thus acidified, diluted with 1% HCl (w/w) to 5 ml. and left overnight. About 0·7 g. of acid-washed kieselguhr is then added, the tube stoppered, shaken vigorously for about 1 min., and then centrifuged for 5 min. The kieselguhr packs well, allowing the brown supernatant liquid to be decanted. Control examination of this liquid by ultra-violet light should show only a greenish-blue fluorescence unmixed with red, thus indicating that the quantity of adsorbent used has been sufficient.

Elution and quantitative determination of porphyrin

The kieselguhr is now washed by stirring up and centrifuging successively twice with 1% HCl (w/w) and once with H_2O.* The porphyrin is then eluted completely by three successive washings with 0·1 *N* NaOH of about 7 ml. each,

* From some urines, washing with 50% acetic acid removes a further small quantity of fluorescent material without loss of porphyrin.

and centrifuging for 15–20 min. The eluates may still contain fine particles of kieselguhr in suspension, but these are disregarded since, when all the eluates are combined, made up to a volume of 25 ml. and left overnight, the turbidity subsides completely, leaving a clear solution which may be pipetted off. From this, a suitable dilution, 1/5 or 1/10, is prepared with 0·1 *N* NaOH, such that the final porphyrin concentration is near the optimal figure of 0·25 μg./ml., and the exact concentration is determined fluorimetrically in the fluorescence comparator [Rimington, 1943].

This determination gives the total porphyrin. Coproporphyrin is conveniently determined, by the acetic acid-ether extraction method and fluorimetric analysis, in a portion of the eluate, or in the urine itself. By subtraction the quantity of uroporphyrin is calculated. It has been found more convenient to elute the total porphyrin with dilute alkali rather than to attempt a two-stage elution and separation of coproporphyrin from uroporphyrin by means of buffers. Errors due to the effect of salts upon fluorescence intensity are thereby eliminated, and, furthermore, quantitative elution by buffers was found to be rather a tedious process, requiring relatively large volumes of fluid.

Recovery of added uroporphyrin was quantitative, and the error of the method, judged by results of repeated determinations, is estimated at less than 10%. The following example may be quoted in illustration:

Urine specimen 26. (Coproporphyrin by ether extraction 147 μg./100 ml.)

		Uroporphyrin mg./100 ml.
(1)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), worked up immediately	0·96
(1*a*)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), worked up immediately	1·08
(2)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), heated on water bath for 1 hr.	1·72
(3)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), left overnight at room temp.	1·67
(3*a*)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), left overnight at room temp.	1·77
(4)	1 ml. urine + 2 ml. H_2O + 1 ml. (= 20 μg.) uroporphyrin III + 1 ml. 5% HCl (w/w), left overnight at room temp. Recovery of added uroporphyrin 109%	
(5)	100 ml. pooled eluates corresponding to 8 ml. urine (= 12 μg. coproporphyrin) extracted by ether, etc. Coproporphyrin in extract 12·4 μg. Recovery 103%	

RESULTS

Excretion of uroporphyrin and coproporphyrin in a case of acute idiopathic porphyria

The patient was a young man aged 19, under the care of Dr Geoffrey Evans of St Bartholomew's Hospital, for whose great kindness in allowing me to examine the urines and publish my biochemical findings, together with relevant data from the clinical record, I am very greatly indebted. He was admitted in April 1942, and presented the typical clinical picture of acute idiopathic porphyria. The prominent features of his condition were malaise and marked asthenia, complicated by peripheral neuritis and muscular wasting. Relapses were characterized by a return of abdominal pain, loss of appetite and nausea. He suffered an attack of pain on 25 May, but thereafter improved until 19 August which marked the onset of another sharp attack.

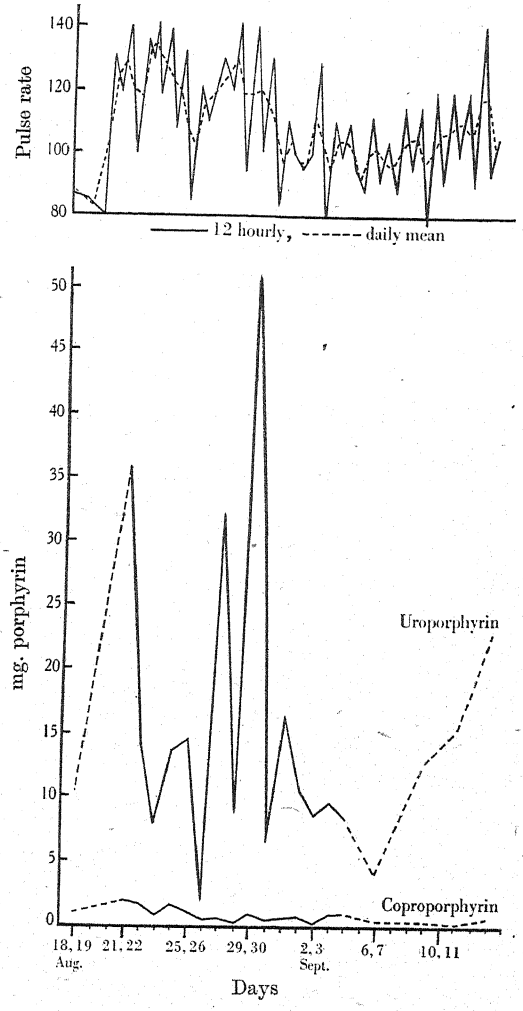

Fig. 1. Daily urinary porphyrin excretion and pulse rate in case of acute idiopathic porphyria. — Consecutive daily determinations. --- Determinations at intervals of 48 hr. or more.

Urine specimens (24 hr. collections, for arranging which I am much indebted to Dr G. Discombe) were examined on the 12/13 August and 21/22 August, and thereafter daily until 5 September. This period embraced a typical attack. As the

patient's condition was improving by 5 September, analyses were performed only at 48 hr. or 72 hr. intervals until 14 September, when the investigation concluded.

Of most urine specimens, 1 or 2 ml. sufficed for the estimation of porphyrin, but of the weaker specimens 3 ml. were required, and in the case of those very rich in porphyrin 0·5 ml. was found to be ample. Table 2 presents the analytical results, together with notes upon the colour of the urines and the clinical condition of the patient. A comparison between the levels of uroporphyrin and coproporphyrin excretion from day to day and the 12-hourly records of the pulse rate (full line) and estimated 24-hourly fluctuations in pulse rate (dotted line) is set forth in Fig. 1.

Table 2. *Case: 'Peter Beresford'. Analysis of urines for porphyrin*

Date	Colour of urine	Total volume ml.	Copro-porph. μg./ 100 ml.	Total copro-porph. mg.	Uro-porph. mg./ 100 ml.	Total uro-porph. mg.	Clinical condition
9/10 June	Nearly black	1420	30·6	0·435	1·9	27·0	Last attack of pain 25 May, condition improving
1/2 Aug.	Light red-brown	—	60·5	—	2·58	—	
12/13 Aug.	Dark	—	64·5	—	1·6	—	
18/19 Aug.	Light brown	1240	76·9	0·954	0·85	10·5	Pulse rate 80
21/22 Aug.	Dark	1915	108	2·068	1·87	35·7	Sudden rise of P.R. to 128 on 20th
22/23 Aug.	Deep brown	1860	100	1·860	0·76	14·14	P.R. 136
23/24 Aug.	Greenish brown	680	124	0·843	1·18	8·0	P.R. fell gradually to 85 on 23rd. Rose again to 110 on 25th; 120-130 on 26th; 140 on 27th a.m.; fell to 95 by p.m.
24/25 Aug.	Golden brown	1160	139	1·612	1·18	13·69	
25/26 Aug.	Red-brown	850	147	1·250	1·69	14·37	
26/27 Aug.	Dark purplish brown	930	68·4	0·657	0·293	2·81	
27/28 Aug.	Dark brown	900	74·4	0·728	3·264	31·93	P.R. 140 on 28th a.m., fluctuating, but fell to 85 by 30th. Patient lost 6¼ lb. since 9 Aug.
28/29 Aug.	Dark brown	182	132	0·251	4·718	8·96	
29/30 Aug.	Very dark	720	130	0·969	6·745	50·25	
30/31 Aug.	Dark brown	815	58	0·487	0·775	6·51	P.R. about 100
31 Aug./1 Sept.	Dark brown	1420	38·7	0·568	1·128	16·58	P.R. rose to 128 on 1st, falling to 80 on 2nd
1/2 Sept.	Dark brown	1100	71·3	0·806	0·954	10·78	
2/3 Sept.	Dark brown	1490	18	0·277	0·547	8·42	Attack subsided, according to P.R., on 16 Sept., when the rate returned to the normal range except for occasional rises to about 130. Steady slow improvement has continued since 18 Sept.
3/4 Sept.	Light brown	1810	49·5	0·923	0·501	9·44	
4/5 Sept.	Light brown	1920	49·5	0·980	0·416	8·24	
6/7 Sept.	Light brown	2290	18	0·425	0·165	3·89	
9/10 Sept.	Light brown	1780	22·7	0·415	0·705	12·90	
11/12 Sept.	Light brown	2120	11·6	0·254	0·716	15·54	
13/14 Sept.	Light brown	2040	35·2	0·739	1·096	23·02	

No interpretation of the clinical findings or discussion of the clinical aspects of the case is intended here; the record of the pulse rate is merely given as an accompaniment to the porphyrin determinations which illustrate the potential usefulness of the kieselguhr method. With such a relatively simple and rapid technique in one's hands, it is now possible to subject the attacks and remissions of acute porphyria to a quantitative examination, and seek to gain further insight into the pathogenesis of this disease. Whilst the correspondence is not exact, it may be said, from an inspection of Fig. 1, that, in general, the quantitative excretion of uroporphyrin follows the excursions of the pulse rate. There is no constancy in the ratio between coproporphyrin and uroporphyrin excreted or between total porphyrin and urine volume (see Table 2), although it would appear that the fluid output modifies slightly the output of pigment.

The urinary porphyrins excreted by this case were identified as uroporphyrin III, octamethyl ester M.P. 258°, and coproporphyrin III, tetramethyl ester M.P. 140°, remelt 163°, together with a small quantity of coproporphyrin I.

SUMMARY

1. Coproporphyrins and uroporphyrins of both series I and III are adsorbed by kieselguhr from acid solution (1 % HCl (w/w)), and a separation of the porphyrins, but not of individual isomers, may be effected by developing the chromatogram with acetate buffer of *p*H 5·25. Uroporphyrins I and III are readily eluted.

2. Improvements in the preparative method of obtaining uroporphyrin from pathological urine are based upon this observation.

3. A quantitative procedure for determining the total porphyrin in urine, and hence the uroporphyrin by subtraction of the independently determined coproporphyrin, is described and its application to the analysis of consecutive 24 hr. samples

from a case of acute idiopathic porphyria, during an attack, is recorded. The provision of a quantitative technique for uroporphyrin determination opens up possibilities for the further study of the relationship between porphyrin formation or excretion and clinical condition in this disease.

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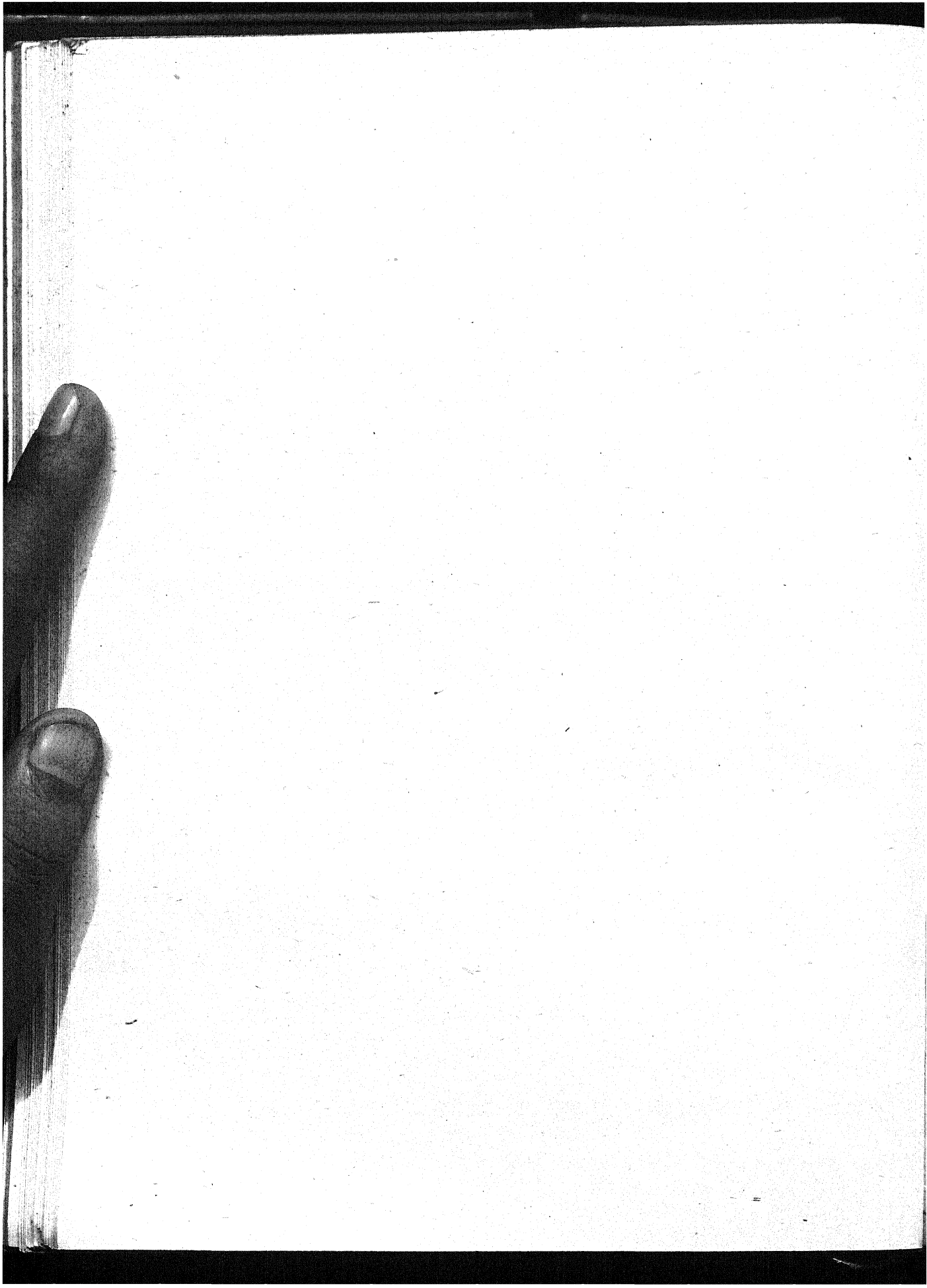

Obituary Notice

ERNEST WALKER (1900–1942)

During August 1942, Dr E. Walker's sudden death came as a considerable shock to all who knew him and occurred while he was still a member of the Committee of the Biochemical Society.

Born in 1900, he was the son of a Liverpool corn merchant, but was left an orphan early; both parents died when he was nine years old. He was educated at Pinner School and the Liverpool Collegiate School, and left at the age of seventeen to join the Army (The Artists' Rifles). Returning from the war, in 1919 he studied chemistry at Liverpool University under the inspiration of Prof. E. C. C. Baly.

In 1920, he came to the Biochemical Laboratory, Cambridge, to work as assistant to Dr R. A. Peters upon problems of chemical constitution and physiological action in relation to Chemical Defence Research. This work was continued after 1923 in the Department of Biochemistry, Oxford, whence he accompanied Dr R. A. Peters and became a member of St John's College. This research formed the basis of his thesis for the M.Sc. Liverpool, and the D.Phil. (Oxford) degree which he took while temporarily employed at the Government Experimental Station at Porton in 1927. During this period, a fundamental contribution on the physical chemistry of mustard gas and related compounds in relation to vesication was published (*Biochem. J.* 1923, **17**, 260, with R. A. Peters). He also developed earlier work by McCleland and Peters upon the toxicity of ter- and pentavalent arsenical compounds to Protozoa (*Biochem. J.* 1928, **22**, 292). Some of the work of this period was inspired by that of Sir F. G. Hopkins upon sulphydryl compounds, particularly his research work upon the fixed —SH group in muscle tissue (with M. Dixon). In especial Walker worked out a nitroprusside test for —S—S— groups, using cyanide, and located the extent of the layer in skin showing the nitroprusside reaction. It was this research which led to the important discovery (independently and about the same time as Voegtlin) of the effect of an —SH compound (monothioethylene glycol) in reversing the action of diphenyl arsenious oxide upon ciliates.

After a temporary interlude at the Experimental Station, Porton, he returned to Oxford in 1928 and proposed at first to qualify in medicine. Subsequently, however, he gave up this plan and settled down as a Demonstrator, occupying the post of Departmental and then University Demonstrator until the end. He also undertook the secretarial work of the Department; here he devoted himself to promoting successfully its efficiency by his continual and unselfish attention to its interests as a whole, and skill in administrative detail. The students owe much to his attention to the smooth running of the classes, as also to his constant interest in teaching fundamentals. The development of the courses for the Supplementary Subject in Biochemistry for the Final Honour School fell to him, and he was responsible for teaching the Microbiological Biochemistry.

In the period from 1928 to 1940, Dr E. Walker published several papers, on a variety of subjects, several with junior pupils and colleagues, though much of his constant labours did not see the light. Becoming suspicious of the Pettenkofer reaction as an indication of the presence of bile salts in normal blood, he found that this was due to cholesterol oleate, which he isolated (*Biochem. J.* 1930, **24**, 1489). With A. R. Armstrong (*Biochem. J.* 1932, **26**, 143) he identified the substance in pregnancy urine giving Knoop's test, as histidine, a piece of work which seems to have been overlooked by some recent workers. Returning to sulphydryl compounds, he studied (with A. Todrick) the effect of allyl isothiocyanate upon cysteine, isolating the compound formed; at the same time and independently of Anson and Mirsky they developed a method (differing from their own) for estimating the sulphydryl groups in protein, which was applied to ovalbumin and albumin, also myosin (*Biochem. J.* 1937, **31**, 297). In his later work he turned to microbiological biochemistry. With F. L. Warren, he worked upon the decomposition of cellulose by Cytophaga I (*Biochem. J.* 1938, **32**, 31). Chemical advances in a difficult field were here made with this organism, which has so unique an appetite for cellulose; characteristic was the realization of the biological importance which may be played by oxycellular mucilage in the humus of the soil. His final publication with W. H. Maguigan (*Biochem. J.* 1940, **34**, 804) was concerned with the sterol metabolism of yeast; this formed part of a more ambitious programme, some of which has not yet been published, and broke new ground of importance. Facts were established about the time of origin of sterol and about precursors. During this war, Walker was engaged upon problems in connexion with the disposal of sewage; but much of his time

latterly was occupied in administrative problems and in the early development of the Oxford Nutrition Survey, which owes much to his wise guidance. Appraising the work as a whole, we realize the careful chemical interest in completion, combined with a wide knowledge and appreciation of biological applications; he had learnt fully the necessary physiology and bacteriology required. It indeed seems that his studies on micro-organisms were only at an initial stage.

The Secretary has told me that as a member of the Committee of the Society, Walker displayed a quiet ability combined with a sense of humour that proved to be invaluable in finding a way through the complicated results of a discussion on procedure or the like. His complete familiarity with the rules of the Society and of syntax alike could on occasions be a little disconcerting but extremely helpful.

Quiet and modest in manner, unfailingly honest in his judgements and dealings with men, he proved a wise, independent-minded, courageous and respected counsellor in the laboratory. He had the capacity for carrying through administrative detail without appearing flurried or perturbed; in part this was due to an unfailing sense of humour which never deserted him, and which so often proved disconcerting. One of his many hobbies was chess playing.

In 1932 he married Dr Vera Reader, whose work upon nutritional problems is well known to biochemists, and is survived by his wife and two children.

R. A. PETERS

The Enzymic Production of Levan

BY S. HESTRIN, S. AVINERI-SHAPIRO AND M. ASCHNER, *Chemistry Department, Cancer Laboratories, and Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem*

(*Received 10 April 1943*)

This paper describes the preparation and study of cell-free levan-synthesizing enzyme systems (levansucrase) from two bacterial species [cf. Aschner, Avineri-Shapiro & Hestrin, 1942]. A particularly convenient source of synthesizing enzyme has been found in a newly isolated non-sporulating bacterium to be referred to as *Aerobacter levanicum*. Recently Hehre has achieved the cell-free synthesis of dextran by the action of an enzyme isolated from *Leuconostoc mesenteroides* [Hehre, 1941; Hehre & Sugg, 1942; Stacey, 1942]. Levan- and dextran-synthesizing enzymes are thus now available in sterile form. The two principal polysaccharides which bacteria form specifically from sucrose may henceforth be included, together with glycogen and starch, in the category of macromolecular materials whose production can be effected *in vitro* at will.

Nomenclature

Beijerinck [1910, 1912] coined the word viscosaccharase in generic designation of enzyme systems which mediate polysaccharide-gum production from sucrose. We propose to designate individual enzymes within this group in each case in accordance with the polysaccharide whose production is catalysed. Thus for levan-synthesizing enzyme: levansucrase; for dextran-synthesizing enzyme: dextransucrase. Since the viscosucrases are very different in specificity from phosphorylase, the enzyme whose activity mediates the production of starch or glycogen, it seems correct to retain for the dextran- and levan-synthesizing systems the nomenclature and classification proposed by Beijerinck. It should not be overlooked, however, that the over-all processes of levan and dextran production fit ill in any recognized class of carbohydrate-mediated reactions. The assignation of the dextran- and levan-synthesizing enzyme group to the sucrases can therefore at best be only tentative.

The enzymes of levan *hydrolysis* are, as will be shown elsewhere, distinct from levansucrase. They belong by definition, together with inulase, to the class of polyfructosidases. They may conveniently be referred to, therefore, as levanases.

EXPERIMENTAL

Bacterial strains used. Three bacterial strains were selected for investigation after preliminary trials with a sucrose-agar medium had indicated their excellence as levan formers. The first was *Bacillus subtilis*, Marburg strain, the levan of which has been the subject of extensive chemical study [Hibbert & Brauns, 1931]. This species is typical of a large group of sporulating bacteria which induce levan deposition in sucrose-agar medium at a distance from the actual growth site of the colony. The second organism selected was an aerobic gas-producing spore former received from the National Collection of Type Cultures under the name *B. polymyxa* (Prazmowski) Migula [*B. asterosporus* (Meyer)] N.C.T.C. no. 4744. It differs morphologically in the mode of its levan production from organisms of the *B. subtilis* type, depositing levan in sucrose medium at the cell-medium interface in the form of a giant capsule. The third test organism was a lactose-fermenting non-sporulating bacterium isolated from a fruit-tree root in Jerusalem. Like *B. polymyxa* it produced levan at the cell-medium interface only. The organism is actively motile, possessing peritrichous flagella. It ferments glucose, fructose, lactose, sucrose, raffinose and mannitol with the production of acid and gas, but ferments glycerol, inulin and levan with pro-

duction of acid only. The gelatine liquefaction test, and the methyl-red and citrate tests were all negative, and the Voges-Proskauer reaction positive. The organism therefore appeared to belong to the *Aerobacter* group. We propose for it the name *A. levanicum*.

Estimation of levan. Levan is defined for the purposes of this investigation as material precipitable by 72% ethanol and hydrolysable to fructose by hot dilute acid of *p*H 2·0. A method was required which would permit a fairly precise assay of levan, as thus defined, to be made in small amounts of fluid when large amounts of sucrose or reducing sugar are also present. A satisfactory method was developed from data given for *Bacillus subtilis* levan by Hibbert, Tipson & Brauns [1931].

The following procedure was adopted: 1·0 ml. test solution is added to 3·0 ml. ethanol in a centrifuge tube of 15 ml. capacity. As a rule all levan present separates immediately as a flocculent sediment. If flocculation is slow, it can be hastened and rendered quantitative by the addition of a drop of 1% $CaCl_2$. The suspension is centrifuged, and the sedimented levan is freed from reducing sugar and sucrose residues by twice-repeated solution in H_2O over a water-bath and precipitation each time with ethanol. Addition of a glass bead to the mixture facilitates these operations. The final sediment is taken up in 3·0 ml. 0·5% oxalic acid and complete hydrolysis effected by heating in a boiling water-bath for 1 hr. Evaporation is restricted during this treatment by placing a glass bulb at the tube aperture. The hydrolysate obtained is neutralized, cleared with $Zn(OH)_2$, and diluted to a suitable volume. The reducing power of the filtrate is estimated by the method of Somogyi. The amount of levan is calculated from the 'glucose value' by multiplying by a factor which allows both for the small difference in reducing power between glucose and fructose, and for the entry of H_2O during hydrolysis. The efficacy of the method was controlled on solutions of levan from *B. subtilis* of known concentration. Assays in the range 40–400 mg./100 ml. yielded results reproducible within about 5 mg./100 ml. Since the method measures ethanol-precipitated labile furanosidic polysaccharide as distinct from less easily hydrolysed pyranosidic polysaccharide, it is of high specificity.

A rapid qualitative spot-test for polysaccharide in the agar gel medium was found useful. It is based on observation of the turbidity change undergone by pieces of agar gel when they are immersed in methanol. If they contain levan they rapidly become milky white in the area of levan deposition.

Estimation of fructose. Fructose was determined by means of the specific reducing action of the keto-hexose on phosphomolybdotungstic acid. Reaction conditions indicated for method (1) of Davidson, Kermack, Mowat & Stewart [1936] were adopted, and the concentration of fructose was estimated colorimetrically against solutions of known fructose concentration similarly treated. Measurements were made in a Pulfrich photometer using filter S 61. Over a wide range of concentration the extinction values were found to be a linear function of the fructose concentration.

Sterility of enzyme reaction mixtures. Numerous sterility controls were carried out. In the experiments with *B. subtilis* enzyme, the danger of contamination was very great and strict asepsis had to be practised at all steps of the test. In the experiments with *Aerobacter* enzyme sterility was successfully maintained by the addition of a drop of thymol in chloroform to the mixtures. Enzyme preparations and reaction mixtures were considered sterile only if direct microscopic examination and growth tests using both heavy and diluted inocula at the beginning and at the end of the experiments gave a uniformly negative result. Experiments in which contamination occurred were rejected.

RESULTS

A. Bacillus subtilis

Production of levan by living cells

Cultures of *B. subtilis*, grown in fluid medium containing sucrose, caused the appearance of a marked opalescence and an increasing viscosity associated with the appearance of a polysaccharide whose behaviour when isolated was that of typical levan. The concentration level of levan in the medium depended on the conditions of the experiment [cf. Cooper & Preston, 1935]. It was particularly high in a medium which ensured a uniform supply of sucrose and a continuous removal, by dilution, of reaction products other than levan. To this end *B. subtilis* was inoculated into buffered 0·25% peptone in phosphate solution contained within a cellophane sac suspended in a large volume of 10% sucrose in 0·25% peptone. After 4 days at 30° the concentration of levan in the internal phase had reached a record level of 12·5%; outside the sac, the levan content remained nil.

In sucrose-2·5% agar medium, growth of *B. subtilis* and similar species is remarkable because of the appearance of a peculiar non-continuous coacervate pattern at a considerable distance from the actual growth site of the colony [Beijerinck, 1912]. Levan powder sprinkled on the sucrose agar diffused through the medium only very slowly and failed altogether to form within it characteristic coacervate patterns of the type observed near *B. subtilis* colonies. There seemed to be strong evidence, therefore, that levan coacervation at a distance from the bacterial colony growing on sucrose agar is due to the activity of a diffusible enzyme within the region involved. Resting cells of *B. subtilis* grown on sucrose medium and suspended in pure sucrose solution synthesized levan actively. In peptone cultures containing levan as sole source of carbohydrate, *B. subtilis* grew satisfactorily with formation of much acid.

Specificity. Corresponding substrate specificity in levan production between the strain of *B. subtilis* here employed and that studied by Harrison, Tarr & Hibbert [1930] was noted. In the experiments with our strain, a resting-cell technique was employed. Mixtures were composed of 0·4 ml. physiological saline containing the thoroughly washed cell crop of 10·0 ml. sucrose-peptone cultures of *B. subtilis* (harvested after 10 days of growth at 30°) and 1·0 ml. of sterile 1·0 *M* solution of sugar in Sörensen phosphate buffer *p*H 6·8. Levan assays were carried out both at the beginning of the experiment and

after 2 days' incubation at 30°. Glucose, fructose, invert sugar, trehalose, maltose, melezitose, and methyl-β-fructofuranoside (syrup preparation after Menzies [1922]) failed to support levan production. Sucrose and its galactoside raffinose, on the other hand, were readily built up into levan. Inulin was fermented by the cells with formation of some reducing sugar. Levan coacervation was not evident, however, in inulin agar on which *B. subtilis* was grown (cf. also *Streptococcus salivarius* [Niven, Smiley & Sherman, 1941]).

The negative findings with methyl-β-fructofuranoside and inulin are in apparent disagreement with a generalization on levan production formulated by Harrison *et al.* [1930]. This states that substances with a fructofuranoside terminal unit, hydrolysable by fructosidases, are suitable indirect substrates of levansucrase activity. Experiments with cell-free enzyme preparations may elucidate this question.

Experiments on cell-free levansucrase from B. subtilis

Sterile Seitz filtrates of *B. subtilis* cultures in fluid sucrose medium failed to produce levan in a detectable amount. It was concluded either that levansucrase in the fluid culture is predominantly endocellular or that it is exocellular but absent from the filtrate because it is adsorbed during filtration on the Seitz filter pad. The situation in sucrose-agar medium is very different. Here levansucrase is actively secreted into the medium and can be effectively separated free from living cells by means of a technique of selective diffusion through agar gel originally invented by Beijerinck [1912].

Pieces of sterile agar medium composed of 0·5 % peptone, 0·1 % K_2HPO_4 (*p*H 7·2), 2·5 % agar and 2·5 % sucrose were laid out on a Petri dish and covered by corresponding pieces of gel medium similarly compounded but without sucrose. The pieces were about 1 cm. square and 3 mm. in thickness. *B. subtilis* was sown in heavy inoculation in the central area of each top piece. After incubation for 24 or 48 hr. at 30°, the top layers were aseptically removed. The sterile bottom layers contained levansucrase.

Pieces of agar containing levansucrase prepared as described were suspended in several volumes of 4 % sucrose solution at 37°. Large amounts of reducing sugar appeared in the medium and there was a progressive increase in turbidity and viscosity with the formation of a non-reducing substance precipitable by 72 % ethanol and hydrolysed in hot dilute oxalic acid with liberation of fructose. After 12 hr. incubation, 300 mg. levan/100 ml. were demonstrable in the fluid phase as against virtually none in the fluid phase of control suspensions consisting of similar pieces of agar in H_2O. The control and test mixtures remained sterile. It was therefore clear that production of levan had been accomplished in the absence of living cells.

Attempts to extract the *B. subtilis* levansucrase from the pieces of agar met with only limited success. Enzyme-containing pieces of agar, prepared as described above, were ground up with 4 % sucrose solution. A particle-free fluid which possessed synthesizing activity was separated from the mixture by centrifugation. After incubation for 24 hr. at 30°, this sucrose eluate contained 61 mg. levan/100 ml.; a boiled sample similarly incubated was found to be almost devoid of levan. In both cases the solution remained sterile.

Diffusion of levansucrase and enzymically induced formation of levan as a coacervate in agar gel. It is evident from the method of preparation that levansucrase is secreted into sucrose agar by growing cells of *B. subtilis*, the secreted enzyme diffusing through the agar. To demonstrate this, a levansucrase-impregnated piece of agar was placed on a similarly sized piece containing sucrose but devoid of enzyme. After 15 min. at room temperature the top piece was removed and the bottom piece, which at this time contained no sign of levan, was incubated at 30° for 24 hr. Although the bottom piece remained sterile, a typical levan coacervate was found throughout its interior at the end of the incubation. Further trials involving several superposed layers of agar suggested that the rate of diffusion of measurable amounts of levansucrase through sucrose agar at room temperature was at least 0·1 mm./hr. (cf. Table 2). These results explain the ability of living *B. subtilis* to induce formation of levan coacervates in agar at a distance from its site of growth.

Effect of sugar on levansucrase production. Presence of sucrose within the growth medium is a prerequisite for production of levansucrase by *B. subtilis*. When this organism was grown on an agar medium devoid of sugar or containing maltose or glucose instead of sucrose, the levansucrase activity of the gel medium was found to be nil. The secretion and probably also the formation of levansucrase by *B. subtilis* in agar medium would appear from this result to be strictly adaptive. Cells harvested from broth cultures actively produced levan when suspended in sucrose solution if the latter had also been present in their growth medium, but produced little if any levan from sucrose when harvested from broth containing invert sugar as the sole carbohydrate.

Stability of the enzyme. The enzyme is thermolabile and is completely inactivated by brief exposure to 100°. Trituration under ethanol and acetone followed by rapid desiccation did not deprive levansucrase-impregnated agar of synthe-

sizing power [cf. Dienes, 1935]. By this treatment the enzyme could be obtained in a form suitable for prolonged storage.

B. Bacillus polymyxa

Production of levan by living cells

The growth of *B. polymyxa* in fluid culture media containing sugar is associated with increases in viscosity and opalescence which are particularly marked when the medium contains sucrose. The cells are found enclosed within a capsule which is large when sucrose, and small when another sugar, e.g. glucose or lactose, is the sole source of carbohydrate. The polysaccharide formed in the sucrose medium was separated by ethanol precipitation after partial removal of cells by centrifugation. After repeated solution in H_2O and precipitation by ethanol, the sticky precipitate was converted into a friable powder by trituration under methanol. In the ease with which it could be hydrolysed, liberating fructose, it resembled *B. subtilis* levan. The polysaccharide formed by *B. polymyxa* from lactose, on the other hand, was of a different type. This product, itself non-reducing, failed to form reducing sugar when heated in $N/100$ acid, and formed reducing sugar only when hydrolysed by hot $2N$ HCl (cf. *Chromobacter viscosum* [Carruthers & Cooper, 1936]).

Discontinuous patterns of coacervated levan were not induced in sucrose agar on which *B. polymyxa* was grown, although the cells themselves were found embedded in a fluid mass from which levan has been isolated in good yield. Tests on pieces of gel from the vicinity of the cell colonies, carried out as described in the section on *B. subtilis* enzyme, failed to demonstrate the presence of levansucrase in the gel medium. Levansucrase is therefore obviously present in *B. polymyxa*, but is endocellular, unlike the enzyme in *B. subtilis* and other levan-producing spore-formers on sucrase-agar medium. Peptone medium containing levan (formed by *B. subtilis* or *B. polymyxa*) as the source of carbohydrate supported active growth of *B. polymyxa* with vigorous production of acid and evolution of gas.

C. Aerobacter levanicum

Levan synthesis with living cells

In a fluid medium with sucrose or raffinose as the source of carbohydrate, *A. levanicum* became heavily encapsulated, and the increase of viscosity and opalescence of the medium was found to be associated with the appearance of a levan. This was not bound to the cells, and was isolated by rapid centrifugation to remove cellular material, followed by precipitation in 75 % ethanol and trituration of the gummy precipitate with absolute methanol. The white powder thus obtained gave viscous opalescent aqueous solutions and yielded fructose on heating in 0·5 % oxalic acid. The cells were not encapsulated nor was the polysaccharide formed when disaccharides other than sucrose and raffinose constituted the source of carbohydrate in the medium. Negative results were also obtained with invert sugar and all common hexoses.

When *A. levanicum* was grown on sucrose agar, discontinuous patterns of coacervated levan were not seen within the gel. The cells themselves, however, were found entirely and deeply embedded in a viscous semi-fluid mass from which polysaccharide material having the properties of levan could be isolated in high yield.

A. levanicum on a medium containing levan as the sole source of carbohydrate grew slowly with some formation of acid but not of gas. Resting cells of *A. levanicum* were active levan producers (Table 3). Strains of *A. aerogenes* ferment sucrose and raffinose but, unlike *A. levanicum*, failed to induce levan production.

Levan synthesis with dried cells

After 2 days' growth at 30°, cells were harvested from 50 ml. nutrient broth containing 4 % sucrose. Immersion of the thoroughly washed cells in ethanol and ether, followed by rapid desiccation *in vacuo*, converted them into a dry sterile powder. When suspended in 2·0 ml. sterile 5 % sucrose at 37°, the cell powder induced formation of levan to a level of 324 mg./100 ml. within 20 hr. When an equal amount of powder was suspended in H_2O instead of sucrose, only negligible amounts of levan were demonstrable after the same period.

Experiments on cell-free levansucrase from A. levanicum

Attempts to isolate levansucrase from *A. levanicum* by methods dependent on ultrafiltration, e.g. the technique of selective diffusion in agar as applied in the case of *B. subtilis*, were negative. Levansucrase in *A. levanicum* appeared to be entirely endocellular. Methods of direct preparation from the cell itself were in this case feasible, since *A. levanicum* was readily killed by treatments which were not destructive of its activity towards sucrose. By ethanol-ether treatment or by autolysis in the presence of thymol and chloroform, sterile preparations of levansucrase could be obtained which possessed good synthetic power and were sufficiently stable for quantitative study.

Levan synthesis with cell autolysate. Preparation in this form was possible by autolysis of the cells in the presence of thymol and chloroform. The following routine was adopted: *A. levanicum* was cultivated in 1 l. conical flasks at 30° in a two-phase

medium consisting of a bottom solid layer of nutrient agar devoid of sucrose, and a thin top layer of 2% aqueous sucrose. After 20 hr. the cells were harvested, rinsed repeatedly in H_2O to free them from medium constituents and levan, and finally left to autolyse at 37° under H_2O containing a little thymol and chloroform. The volume ratio of culture medium to autolysate fluid was 100:1. After 24 hr. autolysis, the suspension was sterile. The dry-matter content of the faintly opalescent supernatant fluid from the autolysate was approx. 0·3%. If properly prepared it was entirely free from visible suspended particles. It contained no levan and did not reduce Fehling's solution. It had little or no protein precipitable by trichloroacetic acid. It contained constituents which reduced I_2 in alkaline medium; they were neither removed by dialysis nor by clearing with $Zn(OH)_2$. The fluid rapidly decolorized alkaline permanganate in the cold.

In sucrose solution buffered at *p*H 5·0, to which a little thymol in chloroform was added to maintain sterility, the autolysate induced progressive changes similar to those described above for *B. subtilis* levansucrase. The solution mixtures, which remained uniformly sterile, showed a progressive accumulation of levan polysaccharide (see Table 1). The rate of reaction was maximal at the outset of incubation, and then fell off rapidly. Within the first hour the fluid autolysate, in a mixture composed as indicated in Table 1, synthesized about 100 mg. polysaccharide/100 ml., i.e. an amount approximately equal to the dry weight of enzyme used. The activity of different lots of enzyme varied however, being dependent among other things on the number of bacterial cells harvested.

Table 1. *Formation of levan, reducing sugar, and associated changes in viscosity, induced by an autolysate of* Aerobacter levanicum *in sucrose solution*

Mixtures: 1·0 ml. supernatant enzyme fluid; 1·0 ml. 15% sucrose; and 1·0 ml. Sörensen Na-citrate buffer *p*H 5·0. Temp. 37°.

Viscosity was determined in an Ostwald viscosimeter.

Incubation time min.	Levan as mg. glucose/ 100 ml.	Relative viscosity	Reducing sugar as mg. glucose/ 100 ml.	Ratio of reducing sugar to levan
0	0	1·00	0	—
120	248	1·06	820	3·3
255	330	1·12	1110	3·4
345	341	1·17	1285	3·8

The changes noted failed to develop in the absence of sucrose, or with boiled enzyme. Dialysis against distilled H_2O left the activity of the autolysate towards sucrose unchanged. The catalytic properties of the cell extracts must be ascribed, therefore, to the action on sucrose of an enzyme system, levansucrase, which is typically thermolabile and non-dialysable.

The amount of reducing sugar liberated is considerably larger than the amount of fructoside material found as polysaccharide (Table 1). In no experiment was the ratio of reducing sugar to levan appreciably less than 3, and it was frequently greater. The ratio of reducing sugar to levan as shown by Table 1 is variable, and rises markedly with increasing incubation time. Both glucose and fructose can be identified in the reducing fraction. Moreover, *Aerobacter* strains other than that here described readily ferment sucrose and raffinose though they form no levan from these substrates. It may be assumed, therefore, that side by side with a levan-forming enzyme there is present in *A. levanicum* autolysate an ordinary invertase. It is still undecided whether the latter is indispensable to the process of levan formation [cf. Owen, 1923; Harrison *et al.*, 1930; Norman, 1937].

Diffusibility of levansucrase from A. levanicum *and deposition of formed levan in agar gel.* Levansucrase of *A. levanicum*, unlike that of *B. subtilis*, is non-diffusible in 2·5% agar gel. An experiment which shows the difference in diffusibility between the sterile levansucrase of the two bacteria strains is given in Table 2. This finding suggests that levansucrases of *B. subtilis* and of *A. levanicum* respectively are of different micellar size.

Table 2. *Diffusibility of levansucrase of* Bacillus subtilis *and* Aerobacter levanicum *in agar gel*

Agent	Levan reaction at various depths of agar gel: 0–2 mm.	2–4 mm.	4–8 mm.	6–8 mm.
B. subtilis:				
Living cells	+ + +	+ + +	+ +	+ +
Levan powder	+ + +	−	−	−
Levansucrase (gel)	+ + +	+ +	+ +	+
A. levanicum:				
Living cells	+ + +	−	−	−
Levan powder	+ + +	−	−	−
Levansucrase (fluid)	+ +	−	−	−
Levansucrase (cell-residue)	+ +	−	−	−

2 mm. layers of 2·5% agar gel containing 4% sucrose were superposed and the source of levan allowed contact with the top layer. Tests for levan were carried out on the different agar layers after 24 hr. incubation at 30°. +, positive levan reaction; −, negative levan reaction.

When *A. levanicum* autolysate is left in contact with sucrose-agar gel under sterile conditions for several days, levan is formed, which is particularly concentrated in the fluid layer immediately above the gel, and is in greater dilution throughout the fluid phase. A very thin layer of levan is deposited

at the gel-enzyme interface, but no levan is found in the agar at depths greater than 1·0 mm. It seems therefore that the conditions of this experiment correspond with those which are associated with capsule formation in living cultures.

Effect of sugar on levansucrase production. In *B. subtilis* the production of levansucrase is adaptive, but in *A. levanicum* its production is constitutive (Table 3). The levansucrase activity of cells from media containing sucrose or invert sugar is seen to be very similar. From media without added sugar, enzyme preparations of lower activity were obtained, probably because cell growth in the absence of carbohydrate was poor.

Table 3. *Effect of sugar on production of levansucrase by* Aerobacter levanicum

Exp.	Synthesizing agent	Sugar in medium	Levan formation from sucrose mg./100 ml.
A	Resting cells	Sucrose	204
		Invert sugar	208
B	Autolysate	Sucrose	140
		Invert sugar	100
		None	34

50 ml. broth, containing 4% of the test sugar (if present), were incubated, after inoculation, for 2 days at 30°. The cell harvest (or the autolysate therefrom) was suspended in 3 ml. 0·9% NaCl and sucrose added to a final concentration of 5%; the mixture incubated for 20 hr. at 37°. In resting-cell mixtures antiseptic was not added. Levan was determined on mixtures cleared by centrifugation. In the absence of sucrose, levan content was nil.

Stability of enzyme. Levansucrase from *A. levanicum* possesses considerable stability and its solutions should prove well suited for chemical manipulation with a view to enzyme purification. Experiments showing the potency of the enzyme solution after storage at different temperatures are summarized in Table 4.

Table 4. *Stability of solutions of* Aerobacter levansucrase

Conditions of storing of enzyme		Activity of enzyme (mg. levan/100 ml. formed in 20 hr. at 37°)
Temp. (°C.)	Time (days)	
4	2	241
	21	243
22	2	248
	6	258
30	2	245
	6	228
37	2	215
	6	153
100	5 min.	0

Mixtures contained 1·0 ml. enzyme fluid; 1·0 ml. sucrose 15%; 1·0 ml. Na-citrate buffer (Sörensen) *p*H 5·0.

It has already been noted that cell-bound enzyme remains active after exposure to sterilizing treatment with ethanol and ether. The soluble enzyme is similarly stable. Its solution added to 3 parts of ethanol, with addition of a drop of dilute $CaCl_2$, deposited a light voluminous precipitate which, after separation by centrifugation and resolution in water, showed a synthesizing activity about one-half that of the original solution. Enzyme powder suitable for prolonged storage has also been obtained by desiccating the autolysate in the frozen state in high vacuum; there was some loss of activity.

Nature of polysaccharide produced by levansucrase from A. levanicum. More than 9 g. polysaccharide material have been prepared in the course of this work by the action of a solution of the enzyme on sucrose under conditions of strict sterility. This material has been converted into a powder by trituration under methanol. The material was non-reducing to Fehling solution, N-free (Lassaigne), not hydrolysed by yeast invertase, and gave no colour reaction with I_2. Its aqueous solutions, in contrast to those of inulin, were markedly turbid and viscous, and did not yield reducing sugar on boiling in neutral solution. It was rapidly converted to reducing sugar by boiling in $N/100$ HCl.

The gum-like material precipitated by ethanol was completely hydrolysed by treatment for 1 hr. at 95° with 0·5% oxalic acid. The total reducing sugar thus liberated and calculated as 'glucose' was shown to be fructose by the specific colorimetric assay of keto-hexoses by means of phosphomolybdotungstic acid. The hydrolysate formed glucosazone (m.p. 210°) with phenylhydrazine. The alcohol-precipitable polysaccharide fraction therefore probably contains only fructose, and, because of the ease of hydrolysis of the polysaccharide, the sugar is probably in the furanose form.

The polysaccharide formed from sucrose by the action of *A. levanicum* autolysate is thus a levan. Whether the enzymically formed product is homogeneous and identical with the *Aerobacter* levan produced *in vivo*, and whether it is identical with other levans (e.g. that from cultures of spore-forming organisms [Hibbert & Brauns, 1931; Hibbert *et al.* 1931], from *Actinomyces* [Veibel, 1938], from certain bacterial plant pathogens [Lyne, Peat & Stacey, 1940], from a grass (*Pea trivialis*) and from barley [Challinor, Haworth & Hirst, 1934], is not known.

SUMMARY

1. A micro-method suitable for the specific estimation of levan in the presence of large amounts of reducing sugar and sucrose is described.

2. Levan production by *Bacillus subtilis* (I), *B. polymyxa* (II), and a newly isolated non-sporu-

lating species referred to as *Aerobacter levanicum* (III) has been studied. All three produced levan from sucrose and raffinose. In I, levansucrase is formed adaptively; in III it is constitutive. In I, growing on sucrose agar, levansucrase is exocellular and diffuses through the medium; in II and III on the same medium, it is endocellular.

3. Levansucrase has been prepared cell-free from III as an aqueous solution, as well as in soluble dry form. The cell-free enzyme systems act on sucrose with rapid formation of levan and of reducing sugar. Certain physical properties of cell-free levansucrase from I and III have been studied.

4. Levan has been prepared in substantial amount as a nitrogen-free powder by the action of sterile levansucrase from III on sucrose. Some properties of the enzymically formed polysaccharide are described.

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Complement Activity and Vitamin C

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In recent years evidence has accumulated that deficiency of ascorbic acid impairs the defensive reaction of the organism towards infection. There have been many attempts to correlate this anti-infective effect more specifically with factors participating in immunological reactions [cf. Perla & Marmorstan, 1941]. The greatest attention perhaps has been paid to the possible correlation between the complement activity of the serum and the vitamin C intake.

Although Zilva [1919, 1936] found no significant change of complement titre in scorbutic guinea-pigs, Simola & Brunius [1933] and Marsh [1936] claimed a lowering of the complement activity with a low intake of vitamin C. Chakraborty [1937] could not confirm the finding that vitamin C deficiency affected the complement titre in guinea-pigs.

More recently, Ecker, Pillemer, Martiensen, Wertheimer & Grandis [1938*a*], Ecker, Pillemer & Wertheimer [1938*b*], Ecker, Pillemer, Griffiths & Schwartz [1939] and Ecker & Pillemer [1940] claim to have established a direct correlation between the complement activity of the serum and its concentration of vitamin C. They based their conclusions on average results without any statistical evaluation of the significance of the means. On the other hand, Maccolini [1939] and Agnew, Spink & Mickelsen [1942] found no correlation between the complement titres of guinea-pigs and their intake of ascorbic acid. In view of these conflicting findings, we have reinvestigated the question.

The method of titrating the complement was based on the determination of 50 % haemolysis, and the results were statistically evaluated. It may be said in advance that no significant changes were found in the complement titres of guinea-pigs at different levels of vitamin C intake.

EXPERIMENTAL

Titration of complement. The end-point of the titration ('complement titre') was taken as the volume of serum, expressed in units of which one unit is equal to 0·0001 ml. of undiluted serum, needed to produce 50 % haemolysis in a standardized system described below. This arbitrary end-point, being on the steepest slope of the S-shaped titration curve, increases the accuracy of the titration [Brooks, 1920; Wadsworth, Maltaner & Maltaner, 1931]. The value so obtained expresses in reciprocal form the complementary activity of serum. Sheep cells derived from one source were used throughout the experiment and were standardized according to the procedure of Herbert [1941].

Blood samples measuring 2–3 ml. were obtained by cardiac puncture without anaesthesia. The serum obtained by clotting was diluted with 0·9 % buffered saline of *p*H 7·4 in a ratio 1:30, and eight tubes were set up containing different amounts of undiluted serum, ranging from 0·025 to 0·002 ml. The volume of all the tubes was adjusted to 0·75 ml. by the addition of buffered saline and 0·5 ml. of a suspension of sensitized sheep red cells was added. It was prepared 15–20 min. before use by mixing equal volumes of rabbit anti-sheep haemolysin and of washed sheep red cells containing 50 mg. haemoglobin/ml. The tubes were incubated for 30 min. in a water-bath at 37°, cooled to 2–4°, and 2 ml. of buffered saline were added to facilitate the subsequent reading. After the mixture had been centrifuged, the percentage of haemoglobin present in the supernatant fluid was estimated colorimetrically as described by Herbert [1941]. The results were plotted on squared paper, the haemolysis-serum volume curve fitted and the value for 50 % haemolysis read by interpolation. The titrations were usually done in duplicate.

Animals. Two series of experiments were performed at different seasons of the year (June–July; Oct.–Dec.). Twenty-two guinea-pigs weighing 300–368 g. were used in the summer experiment. The second series, in winter, consisted of eighteen animals averaging 509 g.

Basal diet. The basal diet contained oatmeal, bran, dried yeast, egg yolk, salt mixture and radiostoleum, with water *ad lib.* The solution of pure crystalline vitamin C was prepared daily and pipetted into the mouths of the animals.

Supplements of vitamin C. As will be seen from Table 1, the eleven animals in group 1 (summer) were maintained without ascorbic acid; animals in group 2 (controls) were given a daily dose of 5 mg. of ascorbic acid plus 15 g. of cabbage.

In the winter experiment (Table 2) the diets of the guinea-pigs in group 3 (five animals), group 4 (seven animals), and group 5 (six animals) were supplemented with 0·5, 1·0 and 10·0 mg. of ascorbic acid, respectively. After 4 weeks the dose of ascorbic acid was interchanged in groups 3 and 5. The animals of group 3 then received 10 mg. and those of group 5 0·5 mg. daily.

Table 1. *Complement titres and weights of guinea-pigs on diets deficient in vitamin C and supplemented with 5 mg. ascorbic acid plus cabbage*

	No.	Ascorbic acid added mg./day	Complement titres units (one unit = 0·0001 ml. of undiluted serum) Week 2nd	3rd	4th	Wt. (g.) Week 0	2nd	3rd	4th
Group 1	1	0	64	60	—	306	348	261	219*
	2	0	81	62	—	327	380	272	237*
	3	0	50	53	—	335	370	373	205*
	4	0	66	46	—	308	334	257	Died
	5	0	66	64	—	312	344	244	188*
	6	0	62	64	—	309	342	257	Died
	7	0	50	72	—	306	338	216	Died
	8	0	53	50	—	368	386	325	224*
	9	0	60	72	—	340	364	258	202*
	10	0	50	80	—	304	324	200	Died
	11	0	66	71	—	330	335	291	192*
		Mean	61	63					
		General mean	62						
Group 2	12	5 mg. + cabbage	60	37	64	335	346	367	372
	13	,,	64	—	—	310	389	Died	—
	14	,,	66	54	60	301	384	426	250
	15	,,	66	61	70	320	382	410	441
	16	,,	72	58	56	308	394	444	490
	17	,,	54	43	50	306	360	366	380
	18	,,	51	59	65	346	386	425	485
	19	,,	55	64	54	343	409	439	495
	20	,,	64	58	66	308	347	354	378
	21	,,	58	63	72	339	386	404	442
	22	,,	71	60	62	318	370	401	432
		Mean	62	56	62				
		General mean		60					

* Animals died on 22nd–25th day of experiment. Weight is that on day of death.

Table 2. *Complement titres and weights of guinea-pigs on scorbutogenic diets supplemented with 0·5, 1·0 and 10·0 mg. ascorbic acid*

Group	Week	Dose of vit. C mg./day	Complement titres units (one unit = 0·0001 ml. of undiluted serum) in animals					Mean	Wt. (g.) of animals				
			No. 23	24	25	26	27		No. 23	24	25	26	27
3	0	0·5	—	—	—	—	—		470	450	375	550	570
	2	0·5	—	—	—	55	78	69	525	485	457	470	386
	4	0·5	92	63	70	57	—		515	483	380	432	Died
	7	10·0	38	49	—	—	—	53	470	480	Died	Died	—
	9	10·0	70	50	—	—	—		450	510	—	—	—

Group	Week	Dose of vit. C mg./day	No. 28	29	30	31	32	33	34	Mean	No. 28	29	30	31	32	33	34
4	0	1·0	—	—	—	—	—	—	—	—	640	570	570	520	530	485	450
	4	1·0	65	92	66	66	82	58	61	70	660	550	580	490	500	595	500
	7	1·0	85	68	51	55	65	36	50	59	665	575	610	580	367	630	505
	9	1·0	63	54	50	49	—	42	45	50	665	610	610	635	Died	625	550
									Mean	60							

Group	Week	Dose of vit. C mg./day	No. 35	36	37	38	39	40	Mean	No. 35	36	37	38	39	40
5	0	—	—	—	—	—	—	—	—	500	460	450	490	525	555
	2	10·0	—	50	—	—	—	—		450	480	500	540	550	600
	4	10·0	57	55	72	67	70	72	61	440	545	510	580	570	575
	7	0·5	—	80	197	49	—	—		470	445	435	585	620*	545*
	8	0·5	46	61	63	—	64*	46*	80	470	460	420	595	650*	520*
	9	0·5	48	50	147	57	60*	—		485	465	380	600	600*	Died

* Guinea-pigs 39 and 40 supplemented with 10·0 mg. ascorbic acid daily throughout.

RESULTS

Exp. 1 (summer). The complement titres (Table 1) of deficient guinea-pigs ranged from 50 to 81 units, with a mean of 61 in the second and 63 in the third week of deficiency. The average of all samples had a value of 62 units. The controls maintained on a supplement of 15 g. of cabbage plus 5 mg. of ascorbic acid showed an average for all samples of 60 units (Table 1).

While the weights of the controls increased steadily, the deficient animals started losing weight in the third week of the experiment and died in the following week.

Exp. 2 (winter). The guinea-pigs in group 3 (Table 2) receiving 0·5 mg. of ascorbic acid daily for the first 4 weeks, showed an average complement titre of 69 units. When the supplement of vitamin C was increased to 10 mg., their titre had a value of 53 units.

Group 4, having a supplement of 1 mg. of ascorbic acid, showed average titres of 70, 59, and 50 units in the fourth, seventh and ninth weeks, respectively. The general mean from all the samples of serum was found to be 60 units. The weight of the animals remained at a steady level or showed a slight increase.

The animals in group 5, supplemented with 10 mg. of ascorbic acid for 4 weeks, had an average titre of 61 units. After changing to a partially deficient dose (0·5 mg.), the average value of the titre was found to be 80 units. The weight of all but one animal increased steadily when adequately supplemented with vitamin C. The gain, however, was less than that of the controls in Exp. 1, receiving 15 g. of cabbage in addition to the 5 mg. of ascorbic acid. When the guinea-pigs of group 5 were put on a partially deficient diet in the fourth week, they either lost weight or remained at an almost steady level.

Statistical calculations

It has been claimed [Ecker *et al.* 1938*b*] that a comparison of complement titres between groups of guinea-pigs on different levels of vitamin C intake is unsatisfactory, and that data from the same animal with various levels of vitamin C intake are preferable. Exp. 2 was therefore performed in such a way as to allow a statistical evaluation on this basis. In addition, the significance of the differences between groups on different levels of vitamin C intake was also calculated. The method of analysis of variance was employed throughout [Fisher, 1938; Goulden, 1939].

Significance of differences between groups 1 and 2. The average values of the complement titres of the deficient guinea-pigs (group 1) did not differ appreciably from the average values in group 2, which had received supplements of cabbage and vitamin C. The general mean (60 units) of all samples collected during a period of deficiency agreed almost exactly with the general mean (62 units) of the control group. This insignificant difference between the two means makes a statistical evaluation almost unnecessary. The mean difference between groups 1 and 2 amounts to $-1{\cdot}76$. When the t test was applied, the difference was found to be insignificant ($t = 0{\cdot}797$, while t tabulated for P 0·05 is 2·23). The standard error was calculated from the mean of titres of guinea-pigs bled on the same day.

Significance of differences between guinea-pigs maintained on doses of 0·5, 1·0 and 10·0 mg. of ascorbic acid. When the means of the complement titres for the different levels of

vitamin C intake were calculated, irrespectively of groups, the following values were found:

Mean of complement titres for

0·5 mg. ascorbic acid daily = 75·8, $s \pm$ 10·16 units
1·0 mg. ascorbic acid daily = 60·3, $s \pm$ 3·19 units
10·0 mg. ascorbic acid daily = 58·6, $s \pm$ 3·03 units

The above values may indicate a slight increase in complement activity with an increase of ascorbic acid in the diet. When, however, the value of F, i.e. the ratio of group mean square to error mean square, was calculated, it was found that $F = 2{\cdot}25$; tabulated F for 5 % point = 3·19. It may therefore be concluded that no significant differences had been established.

Significance of differences within group 3. Complement titres of partially deficient guinea-pigs were compared with those of the same animals in the later part of the experiment, when they were given an adequate amount of vitamin C. The variance ratio was $F = 2{\cdot}62$; tabulated F for 5 % pt. = 19·0.

There is therefore no significant difference between the titres of the same animals on different levels of intake of vitamin C.

Significance of differences within group 4. In this group, which had a supplement of 1·0 mg. of ascorbic acid throughout, the titres obtained at different periods of the experiment (4th, 7th and 9th weeks) were analysed. The variance ratio was $F = 3{\cdot}91$; tabulated F for 5 % pt. = 3·98.

As F found is only slightly below the value of F tabulated, the differences are almost significant. This conclusion is interesting as the complement activity increased slightly during the course of the experiment, which is contrary to the findings reported by Ecker & Pillemer [1940].

Significance of differences within group 5. Although the means of complement titres showed an apparent increase from 61 to 80 units when the dose of ascorbic acid was decreased from 10·0 to 0·5 mg. daily, the variance ratio for these findings was $F = 1{\cdot}08$; tabulated F for 5 % pt. = 4·35.

The change of complement titre is therefore not significant.

Analysis of differences between individual guinea-pigs and error of method. Complement titres of individual guinea-pigs within groups did not vary significantly when their variance was compared with the respective error variance. From duplicate samples the standard deviation of a single titration has been calculated ($s = 2{\cdot}33$; $\bar{x} = 60{\cdot}1$). There is therefore a probability of only 1 in 20 that the titres will be outside the limits of $\pm 8\%$.

DISCUSSION

Experimental precautions were taken to eliminate variables other than vitamin C intake which might influence the result. Also by a proper statistical evaluation we eliminated to a certain extent variations due to causes other than vitamin C intake. Thus we took care to bleed the animals without using anaesthetics, which are believed to increase the concentration of ascorbic acid in the blood [Ecker *et al.* 1938*b*]. We also used the same animals as controls for their subsequent complement titres, by putting each animal as far as possible on two levels of vitamin C. The results, particularly those in groups 1 and 2, show without doubt that when a reliable method of complement titration is used there is little, if any, difference between complement titres of individual guinea-pigs within a group, no matter whether they are maintained on a deficient or an optimal diet. We can confirm the conclusions of Brooks [1920] and Wadsworth *et al.* [1931] that the 50 % haemolysis gives consistent results with a low degree of experimental error [Traub, 1943].

It is evident that we could find no influence of the intake of vitamin C on the complement titre. Only in group 4 were the differences between complement titres almost significant. In this group, the figures refer to samples of blood withdrawn from the same animals at different times of the experiment. It may, however, be noted that the complement activity of the sera was either unaltered or improved slightly, quite contrary to expectation based on the findings of Ecker *et al.* [1938*a*] and Ecker & Pillemer [1940]. The guinea-pigs had a supplement of only 1 mg. of ascorbic acid, which certainly was not sufficient to maintain optimal growth. If vitamin C influences complement titres in the way claimed there should therefore have been a decrease of complement activity in this group.

The agreement between the average complement titres of group 2 (Exp. 1) and those in Exp. 2 seems of interest. The animals in group 2 were maintained on a diet supplemented with cabbage as well as an adequate dose of ascorbic acid, and thus received other essential dietary factors [cf. Kohler, Elvehjem & Hart, 1938]. The influence of these could be observed from their improved gains in weight. Nevertheless, their complement titres did not differ from those of the guinea-pigs which received an adequate supplement of vitamin C alone (Exp. 2). Apart from this, although the two experiments were performed in the summer and winter months respectively, no seasonal influence, such as has been claimed, could be observed.

The weights of the guinea-pigs given in the tables show conclusively the influence of the different levels of vitamin C intake. Also, post-mortem findings confirmed the presence of scorbutic changes in the deficient animals.

Our conclusion that the state of nutrition with regard to vitamin C has no influence upon the complement activity of serum is in agreement with results of Crandon, Lund & Dill [1940] in human scurvy. Spink, Agnew & Mickelsen [1942] and Feller, Roberts, Ralli & Francis [1942] arrived at similar conclusions. Further indirect evidence of the lack of positive correlation between vitamin C and complement activity is the finding that although newborn infants have a higher vitamin C concentration in plasma than their mothers [Mindlin, 1940] they have a significantly lower complement activity [Traub, 1943].

Although we could not find any relation between complement and vitamin C, we do not wish to draw any conclusions as to the possible influence of the vitamin on other immunological processes. The exact significance of complement in immunology is in any case still uncertain.

SUMMARY

1. Complement titrations, using 50 % haemolysis as the end-point, were performed on forty guinea-pigs maintained on different levels of intake of vitamin C, and the results were evaluated statistically.

2. No significant change in complement was found to occur in guinea-pigs partially or completely deficient in vitamin C.

3. Addition of cabbage to the diet did not result in any change in the complement titre as compared with that of guinea-pigs whose diet was supplemented with adequate amounts of synthetic vitamin C.

4. The significance of these results is discussed.

We wish to thank Dr L. J. Harris, at whose suggestion this work was undertaken, for his valuable advice and interest throughout the experiment, Prof. H. R. Dean for his interest and facilities for work in his Department for one of us, Dr J. O. Irwin for his criticism of the statistical calculations, and Mr S. Impey for his help in the care of the experimental animals.

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Antigenic Properties of Hyaluronic Acid

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Hyaluronic acid has been shown by Meyer and colleagues [Meyer & Palmer, 1936; Meyer, Smyth & Dawson, 1939; Meyer & Chaffee, 1941] to occur in the vitreous humour, synovial fluid, skin and umbilical cords of various mammals and by the author (unpublished) to occur in the lung tissue. Although the detailed structure of this substance has not been worked out, the work of Meyer and his collaborators [reviewed by Meyer, 1938] has shown hyaluronic acid to be a polysaccharide of high molecular weight, composed of units containing one residue of N-acetyl glucosamine linked with one of glucuronic acid. Existing evidence suggests that specimens of hyaluronic acid prepared from different sources are chemically identical. A polysaccharide which is probably identical has been obtained from capsulated streptococci of groups A and C by Kendall, Heidelberger & Dawson [1937] and by Seastone [1939]. The capsules of these organisms are destroyed by an enzyme—hyaluronidase—obtained from other strains of streptococci or from various other sources [McClean, 1941]. Their presence is associated with the virulent stage of the organisms, but when either whole organisms or purified capsular material were injected into rabbits they failed to give rise to detectable circulating antibodies against the capsules [Seastone, 1939].

In view of these results it seemed of interest to investigate more closely the antigenicity of hyaluronic acid, since not only would specific antisera to this substance be of diagnostic value and possibly of therapeutic use, but the question of antibody production in this instance has a general bearing on theories of antibody formation. It was known (unpublished information from Dr W. T. J. Morgan), that combination of hyaluronic acid with the specific protein obtained from *Shigella dysenteriae* by Morgan & Partridge [1940] was ineffective in conferring antigenic properties, although with other polysaccharides [Morgan & Partridge, 1940; Partridge & Morgan, 1940, 1942] this method is very successful. Recourse was therefore had to the method used by Goebel & Avery [1931] in their work on the artificial coupling of type III *Pneumococcus* polysaccharide to protein by means of a *p*-aminobenzyl ether linkage. The method of Goebel & Avery was not directly applicable to hyaluronic acid since it was found that the number of *p*-nitrobenzyl ether groups introduced varied with the proportion of *p*-nitrobenzyl bromide to hyaluronic acid used, and the different products had very varied solubilities in H_2O and in organic solvents. It may be assumed that the product containing the minimum number of introduced groups is to be preferred, provided that there are enough for effective coupling, since extra groups may mask or modify a weakly antigenic grouping in addition to conferring their own antigenic specificity. A substance has now been prepared containing approximately one *p*-aminobenzyl group per glucosamine residue. It was coupled to horse serum albumin and to normal rabbit serum, the former being used for immunizing and the latter as test antigen, thereby eliminating the effects of non-specific antibodies.

EXPERIMENTAL

(*a*) *Chemical*

Hyaluronic acid. The starting material was the potassium salt of hyaluronic acid prepared from human umbilical cords, purified by repeated extraction with 90% phenol solution and by repeated precipitation from aqueous solution at *p*H 9–10 by 1¼ vol. of ethanol saturated with K acetate. It contained only 75% of the theoretical glucosamine content as determined by Hewitt's modification [Hewitt, 1938] of the method of Morgan and Elson, and was contaminated with finely divided insoluble haematin derivatives extracted with the hyaluronic acid from the cords. These impurities were very difficult to separate from the highly viscous hyaluronic acid without destroying its viscosity. They were, however, readily removed by centrifugation after treatment with *p*-nitrobenzyl bromide as shown below. (Later this work was repeated on a smaller scale using the pure K salts of hyaluronic acid as starting material, with similar results.)

Aminobenzyl ether of hyaluronic acid. To 150 ml. of a 1% solution of hyaluronic acid was added 0·8 g. of finely powdered *p*-nitrobenzyl bromide. The mixture was heated to 100° on a water-bath and 0·75 ml. 20% NaOH added drop-wise and with vigorous stirring during a period of 15 min. Heating was continued for a further 30 min. and the material then neutralized with a drop of acid, steam-distilled to remove traces of *p*-nitrobenzyl alcohol, and centrifuged to remove insoluble matter. The clear solution was dialysed against 3 l. of distilled H_2O and was finally dried *in vacuo* from the frozen state, to give 0·7 g. white, very hygroscopic material. Analysis on dry basis: N = 4·65%; glucosamine = 38·5%; glucosamine/N ratio = 8·4; ash negligible. $[\alpha]_D^{20°} = -58°$. Viscosity of a 1% solution in a crude Ostwald viscosimeter was $9·5 \times H_2O$. (Theory for mono-nitrobenzyl derivative of hyaluronic acid: N = 5·25%; glucosamine = 33·6%; glucosamine/N ratio = 6·4.)

The solution of the *p*-nitrobenzyl ether was reduced with saturated $Na_2S_2O_4$ solution at 50°, the reaction being maintained at approximately *p*H 7, more $Na_2S_2O_4$ being added until the solution bleached litmus paper. It was then dialysed against distilled H_2O until the dialysate gave no yellow colour with Ehrlich's reagent, indicating that the diffusible amino-groups had been removed. The contents of the dialysis bag still gave a definite yellow colour with Ehrlich's reagent. The solution was evaporated to small volume *in vacuo*, and dried *in vacuo* from the frozen state to yield 0·6 g. of a very pale yellow hygroscopic aminobenzyl ether of hyaluronic acid. Two samples prepared independently from separate lots of hyaluronic acid gave the following analyses on a dry basis. *Sample 1:* N = 4·75%; glucosamine = 40%; glucosamine/N ratio = 8·4; $[\alpha]_D^{20°} = -40°$; viscosity of 1% solution = $8·5 \times H_2O$. *Sample 2:* N = 5·00%; glucosamine = 40%; glucosamine/N ratio = 8·1; $[\alpha]_D^{20°} = -65°$; viscosity of 1% solution = $8·6 \times H_2O$.

Theory for mono-aminobenzyl ether of hyaluronic acid is N = 5·4%; glucosamine = 34·5%; glucosamine/N ratio = 6·4. These analyses, although in fair agreement with one another, do not exactly conform to those expected if one aminobenzyl group is coupled to one hyaluronic acid unit. A compound containing 0·66 aminobenzyl groups per hyaluronic acid unit could theoretically give the following analysis: N = 4·9%; glucosamine = 37·5%; glucosamine/N ratio = 7·7, which is in close agreement with the figures obtained. In view of the present uncertainty concerning the detailed structure of hyaluronic acid no reason can be given why, under the conditions used, there should be formed a compound which appears to consist of two aminobenzyl groups per three hyaluronic acid units, nor even whether it is a definite compound. No additional information can be obtained from values for the specific rotation, as these vary from preparation to preparation of hyaluronic acid.

The compound was acted on by testicular hyaluronidase to yield a substance giving the colour reaction for N-acetyl glucosamine. In 1% solution it gave also a definite yellow colour with Ehrlich reagent, and after diazotization followed by removal of excess HNO_2 with ammonium sulphamate, a good pink colour was given with a 0·5% solution of N-1-naphthyl ethylenediamine, indicating the presence of an aromatic NH_2 group. In order to rule out the possibility that the yellow colour given with Ehrlich's reagent might be due to a mixture of unchanged hyaluronic acid and traces of *p*-aminobenzyl alcohol, the substance was extracted with acetone, dioxan and glacial acetic acid without, however, removing any substances giving the Ehrlich colour reaction.

Coupling of the aminobenzyl ether of hyaluronic acid to protein. On the assumption of 1 amino-group per glucosamine residue, 0·5 g. of the aminobenzyl ether of hyaluronic acid preparation was diazotized at 0° with 2·2 equivalents of *N* HCl and 1·0 equivalent of *N* $NaNO_2$. Stirring was continued at 0° for 30 min. and the solutions carefully neutralized to phenol red. The solution gave a strong red colour with alcoholic β-naphthol.

The proteins used were horse-serum albumin and whole-rabbit serum, in the proportions of 3·6 g. protein (calculated from the N content) to 1 g. of the compound. The protein solution (*ca.* 2%) was kept chilled and stirred, and the diazotized compound was added slowly, together with sufficient NaOH to maintain the solution just alkaline to phenolphthalein. Stirring was continued for 1 hr. and sufficient 10% trichloroacetic acid then added to give maximum precipitation. The precipitate was spun down, separated and redissolved in H_2O with a minimum of NaOH. After two or three reprecipitations the supernatant was no longer yellow. The coupled protein was finally made up to about 2% in 0·9% NaCl and dialysed against 0·9% NaCl until it was free from CCl_3COOH. A similar procedure was used in coupling with rabbit serum, but here precipitations of the coupled protein with CCl_3COOH proved to be incomplete and uncoupled material was removed by prolonged dialysis against 0·9% NaCl.

The final products gave pale yellow solutions, and different preparations contained from 5 to 9% glucosamine, calculated from the glucosamine/N ratio. The original serum albumin contained 1·5% glucosamine, which suggests that 7·5–17% of hyaluronic acid had been added by the coupling.

(*b*) *Serological*

Immunization. Three rabbits were immunized with the horse serum-hyaluronic acid preparation. The first course consisted of 30, 60 and 120 mg. injected subcutaneously at weekly intervals. After a week's rest the animals were bled. The second course consisted of four intravenous injections of 20 mg. at weekly intervals, and the animals were bled out 10 days later. None of the animals showed any ill effects during immunization, nor any changes post-mortem such as might indicate antibody formation against a common constituent of the tissues.

Technique of tests on antisera. Precipitin tests were carried out in bulk using constant serum (1:5 dilution) and fivefold dilutions of antigen.

Complement fixation was carried out using two minimal haemolytic doses (M.H.D.) of complement, and the same concentrations of serum and antigen as in the precipitin tests.

Agglutination was tested on a washed suspension of cells from a young culture of a capsulated group C *Streptococcus* whose capsules were known to be dissolved by hyaluronidase. Capsular swelling was tested by incubating equal volumes of serum and streptococcal suspension at 37° for 10 min. and then viewing in wet preparation against a background of India ink.

RESULTS

The results with the serum obtained after the first and after the second course of immunization were substantially the same. Each serum was tested separately, but all behaved similarly. The results are shown in Table 1.

Table 1. *Serological tests on sera of rabbits immunized with hyaluronic acid coupled with horse-serum albumin, against various test antigens*

Test antigen	Precipitin titre	Complement fixation titre
Horse-serum albumin coupled with hyaluronic acid	1 : 10,000	1 : 1000
Whole rabbit serum protein coupled with hyaluronic acid	Nil	Nil
Normal horse-serum albumin	1 : 10,000	1 : 1000
Normal whole rabbit serum protein	Nil	Nil

The positive reactions are evidently due to antibodies against the horse-serum moiety of the antigen, and not against the specific coupled hapten group. No attempt was made to see whether the uncoupled substances would inhibit the reaction between the coupled proteins and homologous antisera. There was no evidence of agglutination nor of capsular swelling in the tests with *Streptococci*.

DISCUSSION

The retention of viscous properties and the hydrolysis by testicular hyaluronidase argue that the structure had not been much altered, and yet it is clear from the above results that even when chemically coupled to a protein, hyaluronic acid is unable to cause antibody formation in rabbits. In this it differs from various chemically similar muco-polysaccharides such as those from pneumococci, from *Shigella dysenteriae*, or the blood group 'A' antigen, but resembles chondroitin sulphuric acid and glycogen, which are also common natural constituents of the mammalian body. The reason for this difference is unexplained. There may be chemical considerations which make the former class of substance able to influence immune serum protein production, while the latter class is unable, but it seems easier to suppose that the second class are of such wide distribution in mammalian tissue that they occur naturally at the sites of antibody production, and therefore exert no different or additional influence when supplied by artificial means.

SUMMARY

Hyaluronic acid coupled chemically to protein by azobenzyl ether linkage does not act as a specific hapten when injected into rabbits.

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The Excretion of Ethylquinone by the Flour Beetle

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While studying inert dust insecticides we observed that the red rust flour beetle (*Tribolium castaneum*) made a brown stain when kept in contact with various fine white powders such as amorphous precipitated silica. When placed on these dusts and kept in dry air the insects did not move about in the material after the first few hours and died after about 2 days. The brown stain developed uniformly round the insect and became visible after the first day, but did not reach its maximum strength until about the sixth day, i.e. long after the insect had died. A similar but smaller stain was produced when dead insects were placed on the powder.

The closely related insect, *Tribolium confusum*, is known to give off an excretion which causes heavily infested flour to become pink and quite unpalatable, and which Payne [1925] found to exhibit aldehyde reactions. Chapman [1926] observed that the same beetle, when stimulated, gave off a nose-irritant gas which turned flour pink. He examined the effect of this gas on the pupae of the beetle and found that the subsequently metamorphosed adults were abnormal. Roth & Howland [1941] passed dry air over *T. confusum*, and isolated the excretion as yellow-brown needles which turned red and eventually blue, even when kept in solid CO_2. At room temperature a gas was evolved of quinone-like odour which was capable of oxidizing HI to I_2. These authors found that the last instar larvae, prepupae and pupae of the insect all metamorphosed into abnormal adults after exposure to this gas.

EXPERIMENTAL

Isolation of the excretion from T. castaneum

The method used for isolating the excretion from *T. castaneum*, based on that used by Roth & Howland [1941] for *T. confusum*, was as follows: About 500–1000 insects (a volume of about 5 ml.) were collected from the insect cultures by taking advantage of the negative geotropism of the adult beetles, sifted free from flour and introduced into a small distilling flask. A stream of dry air was then passed through the flask for about 6 hr., during which time the insects were cooled from 35–40 to 0° for about 20 min. every 2 hr. The excretion was condensed in an ethanol-CO_2 trap, yielding about 0·5 mg. of long, needle-like yellow crystals together with some ice, from the H_2O also given off by the insects. The yield was considerably reduced if the insects were not completely free from flour and larvae, etc. The insects were returned to flour for 3 days and then the procedure was repeated, giving a somewhat smaller yield. After three or four such operations the insects were very feeble and yielded little or no excretion. Many were dead, due, probably, to desiccation. Attempts to separate the H_2O from the excretion by drying with P_2O_5, $CaCl_2$, Na_2SO_4 or $Mg(ClO_4)_2$ yielded a reddish 'polymer' of the excretion on the surface of the drying agent, even when the drying tubes were warmed to 40°.

In view of the small yield per insect (about 0·1–0·2% of the initial body weight) the excretion from some 5000 beetles was necessary for the investigation outlined below.

RESULTS

Properties of the excretion from T. castaneum

The yellow crystals, separated from the H_2O on a cooled porous tile, had a nose-irritant quinone-like odour and were slightly lachrymatory. They were slightly soluble in H_2O, but readily so in ethanol, benzene, chloroform and other organic solvents. The substance was fairly volatile at room temperature and rather labile when first isolated, turning to a red-brown on standing overnight, even at 0°. However, drying increased the stability. The aqueous solution was neutral, reduced Tollen's reagent, liberated I_2 from HI, and gave a brown-black colour with warm dilute alkali. The substance was very sensitive to alkali, but comparatively stable to acid.

No colour was produced with conc. HCl or with $FeCl_3$. When SO_2 was passed through the aqueous solution the yellow colour was discharged and a black precipitate was formed, which later dissolved. N, S, P, and halogens were absent, and tests for aldehyde, ketone, alcohol and similar groups were negative. This chemical evidence identified the compound as a quinone. The m.p. of the dry crystals was 23°, but when the dry substance was allowed to stand at room temperature for several hours the m.p. rose to about 32°.

Preparation and properties of the hydroquinone. The excretion product was reduced immediately after isolation by a solution of $NaHSO_3$ sat. with SO_2. This solution was extracted three times with ether and the combined ether extracts were washed with $NaHCO_3$ and then with H_2O. The ether-soluble fraction was dried azeotropically with benzene, and recrystallized from benzene-ligroin mixture. In this way about 8 mg. of white crystals were obtained, melting at 97–98°. Analysis (Mr W. F. Boston) showed C = 68·8; H = 7·0 % (cf. toluhydroquinone C, 67·7; H, 6·45 %; ethylhydroquinone C, 69·5; H, 7·2 %). A further quantity of this hydroquinone, prepared from an additional supply of insects, melted at 101–102° after 4 crystallizations from benzene-ligroin mixture, but there was insufficient material to continue the purification.

An aqueous solution of the hydroquinone was titrated electrometrically with $N/25$ $Ce(SO_4)_2$ (standardized by a similar titration against pure hydroquinone). Three determinations were carried out on about 1·5 mg. with an error of less than 1 %. The oxidation equivalent weight thus determined was 67. (Theoretical values: toluhydroquinone 62; ethylhydroquinone 69.) Pure ethylhydroquinone gave an equivalent of 70 when titrated in this way.

The m.p.'s, however, of both the insect quinone itself, and of the hydroquinone produced therefrom, differ considerably from those recorded for pure ethylquinone and ethylhydroquinone, but these did not give depressions when admixed with authentic ethylquinone and ethylhydroquinone respectively, whereas there were marked depressions with other homologous members of the series, prepared by standard methods [Johnson & Hodge, 1913; Gulland, 1932; Cook, Heilbron & Lewis, 1942].

Properties of the excretion from T. confusum

A similar examination has been made of the excretion of *T. confusum*. This seems also mainly to be ethylquinone. The yellow crystals obtained had the same m.p. and other properties as the excretion from *T. castaneum*. No volatile excretion could be isolated in the same way from the meal worm beetle (*Tenebrio molitor*) or from the saw-toothed grain beetle (*Oryzaephilus Surinamensis*).

The ultra-violet absorption spectra of some quinones and hydroquinones

While many *p*-benzoquinones have been isolated from natural sources in recent years, descriptions of their ultra-violet absorption spectra are scanty. The naphthaquinones [Morton & Earlam, 1941] have been more fully covered.

Previous determinations of the value of $\lambda_{max.}$ for *p*-benzoquinone itself have in general been in good agreement with each other, but there is a confusion as to the value for toluquinone in ethanol [cf. Lifschitz, Lourie, Zimmermann & Huininck, 1924; Light, 1926]. This confusion probably results from the labile character of mono-substituted alkylquinones in hydroxylic solvents. Thus, the absorption spectrum of toluquinone in ethanol rapidly changes (Table 1) on standing for only a day at room temperature in diffuse daylight.

Table 1. *Ultra-violet absorption spectrum of toluquinone in ethanol*

Time after making up solution	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
ca. 1 min.	245	17,600
1 day	256	8,300
7 days	265	6,350
	228	3,650
	(inflexion) 290	2,000

The data in Table 2 were therefore obtained immediately after the solution in ethanol of the freshly sublimed quinone had been made up.

Table 2. *Ultra-violet absorption spectra of freshly prepared quinones*

Quinone	Solvent	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
Benzo-	Ethanol	242	13,800
Tolu-	Ethanol	245	16,000
Ethyl-	Ethanol	246	16,500
Ethyl-	*n*-Hexane	246	14,800
Thymo-	Ethanol	255	20,600
Insect-	Ethanol	245	—

$\lambda_{max.}$ increases with increasing substitution of the quinone chromophore, an observation in good agreement with previous experimental results with analogous chromophores [cf. Woodward, 1941; Gillam, Barker & Evans, 1940; Gillam & Evans, 1941]. The spectrum of the insect quinone ($\lambda_{max.} = 245 m\mu$) is that of a mono-substituted benzoquinone. This was confirmed by a comparison (Table 3) of the absorption spectrum of the hydroquinone prepared from the insect quinone with the absorption spectra of a number of mono-substituted hydroquinones. All determinations were carried out in ethanol.

Table 3. *Absorption spectra of hydroquinones in ethanol*

Hydroquinone	$\lambda_{max.}$ mμ I	$\lambda_{max.}$ mμ II	$\lambda_{min.}$ mμ I	$\lambda_{min.}$ mμ II	$\lambda_{inflex.}$ mμ	Remarks
Tolu-	228	293	224	251	None	Two maxima
Ethyl-	None	292	None	*ca.* 250	*ca.* 231	—
n-Propyl-	None	295	None	*ca.* 253	*ca.* 228	Shift of maximum to longer wave-length
Insect-	None	292	None	252	*ca.* 228	—
Hydroquinone	$\epsilon_{max.}$ I	$\epsilon_{max.}$ II	$\epsilon_{min.}$ I	$\epsilon_{min.}$ II	$\epsilon_{inflex.}$	Ratio E *ca.* 290 mμ to E at *ca.* 220 mμ
Tolu-	4750	3950	4500	800	None	0·83
Ethyl-	None	3500	None	—	3850	0·74
n-Propyl-	None	3400	None	—	4800	0·71
Insect-	None	—	None	—	—	0·69

DISCUSSION

The evidence leaves little doubt but that the main part of the *Tribolium* excretion is ethylquinone. The other material present in the excretion is probably more volatile than ethylquinone (since the m.p. of the dry insect excretion rises on standing in the air without apparently 'polymerizing'), has a lower C content and therefore a higher O content, has a lower equivalent with respect to $Ce(SO_4)_2$ oxidation and is probably reduced by SO_2; hence it cannot be a simple *p*-benzoquinone derivative.

The excretion of ethylquinone by *Tribolium* is undoubtedly the cause of the pink colour of 'conditioned' flour, the production of the colour being due to the reaction of simple *p*-benzoquinones with amino compounds such as the protein in flour [cf. Gulland, 1932; Fujita & Kodama, 1935; Cooper & Nicholas, 1927]. 5 mg. of toluquinone added to 10 g. of fresh flour rapidly causes the latter to turn pink and to take on the appearance of 'conditioned' flour. Ethylquinone and other simple quinones also cause stains, when placed on amorphous precipitated silica, similar to those caused by the insects themselves. The stain is apparently due to the 'polymerization' of the quinone [cf. Erdtman, 1933].

Apart from the complex anthraquinone derivatives obtained from *Coccus laccae*, *C. ilicis* and *C. cacti* [see Todd, 1941], ethylquinone appears to be the first *p*-quinone to be isolated from an insect source. This is surprising in view of the abundance of *p*-quinones as metabolic products of fungi.

No definite function can at present be ascribed to the ethylquinone excreted by *Tribolium castaneum*, but it is perhaps significant that ethylquinone is a strong spermicide, at least for guinea-pig sperms [Gulland, 1932], and it may well play a part in the remarkable population control in crowded cultures of *Tribolium* [e.g. Park, 1936; Crombie, 1942].

SUMMARY

1. The main part of the volatile excretion of *Tribolium castaneum* has been identified as ethylquinone. The same substance was obtained from *T. confusum*. While, owing to the very small amount of material available, pure ethylquinone could not be isolated, the evidence collected appeared conclusive.

2. The ultra-violet absorption spectra of a number of alkyl-substituted *p*-quinones and hydroquinones are recorded.

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The Effect of National Wheatmeal on the Absorption of Calcium

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When McCance & Widdowson [1942] decided to investigate the effect of brown bread on the absorption of calcium and other minerals from the human gut, the Ministry of Food had not yet announced their intention to introduce a wheatmeal of 85% extraction. The experiments of McCance & Widdowson [1942] were carried out on flours of 92% ('brown flour') and of 69% ('white flour') extraction. Although their data, by way of interpolation, permit an approximate estimate of the properties of a flour of 85% extraction, it seemed desirable, when the national wheatmeal of 85% extraction was adopted, to extend the work to this flour. We undertook such an investigation at the suggestion of Dr McCance. The behaviour of Ca only was investigated, this being representative, as well as from the practical point of view probably the most important of the minerals whose absorption is affected by substances of the outer layers of the wheat grain.

EXPERIMENTS

The experiments were carried out on six volunteers. Their ages and weights are shown in Table 1. They were men of military age who had been put on the register of conscientious objectors and who had originally volunteered for experiments on scabies [see Mellanby, 1941] which were conducted concurrently with the dietary experiments.

Table 1. *Weights and ages of the volunteers*

		Body weight stripped (kg.)		
No.	Age years	At start	After 4 weeks on National Wheatmeal	4–6 weeks later (after 'white' flour regimen)
1	27	66·0	65·4	67·7
2	31	62·8	62·9	63·8
3	30	56·2	56·2	56·9
4	20	60·2	60·8	60·4
5	30	53·8	53·8	54·7
6	23	50·2	52·2	51·8

The dietary and analytical arrangements were essentially the same as those elaborated and described by McCance & Widdowson [1942]. Data concerning one of the consignments of wheatmeal used have been published elsewhere [Krebs & Mellanby, 1942]. The 'white' flour used came from a different grist and was of 75% extraction. Dr Widdowson determined the phytic acid content of samples of the wheatmeal flour and of the bread, and found 140 mg. phytic acid P/100 g. flour, and 41·4 mg. phytic acid P/100 g. bread. The H_2O content of the bread varied between 35 and 38%.

An adverse effect on the Ca balance of phytic acid-containing cereals can be expected only if the Ca content of the diet is relatively low and the cereal content relatively high. In choosing the diet the aim was therefore to keep the Ca content near to the minimum human requirement of 0·55 g./day [Leitch, 1936–7] and to include at least 500 g. wheatmeal flour (dry wt.)/day. Preliminary experiments taught us that the Ca content of the usual diet of the volunteers (during June and July 1941) was above 1 g./day, and that very considerable restrictions had to be imposed in order to reduce the Ca content of the food to the minimum requirement of 0·55 g., namely, (1) complete omission of cheese, (2) restriction of the milk to about 100 ml./day (including milk used in cooked dishes), (3) omission of the vegetables and fruits containing more than 50 mg. Ca/100 g. (e.g. spinach, spinach beet, cabbages, broccoli tops, leeks, spring onions, turnips, water cress, rhubarb, raisins, dried figs), (4) omission of tinned fish (containing softened bones). Further restrictions were due to rationing which allowed approximately 200 g. meat (on the plate), 225 g. fat, 225 g. butter and 123 g. bacon/week. With these restrictions the diet contained during August and September 1941 on average 0·55 g. Ca/day, of which 0·12 g. was derived from milk, 0·12 g. from bread, which had not been fortified by the addition of Ca, and the remaining 0·31 g. from other sources, mainly vegetables. This last source of Ca seems to be subject to very considerable seasonal variations. It stayed at the above-mentioned level of about 0·3 g. during August and the first half of September, but fell later in September and during October to about 0·2 g./day. During the later period it was necessary to raise the milk content of the diet in order to maintain the Ca supply of 0·55 g./day.

Of the flour an average of 70 g. (dry) was eaten in the form of cooked dishes, and the rest in the form of bread. To make the large quantity of bread—600–900 g./day—palatable it was necessary to supply an extra jam ration of 1 lb./head/week.

The average total calorie intake was about 4100/day. Of these 2200 came from flour.

Our main experiment, carried out after preliminary trials, consisted of two periods. During the first, lasting 4 weeks from the middle of August to the middle of September 1941, national wheatmeal was the staple food; the second was a control period, lasting 4 weeks (in one case 3 weeks) between the end of September and early November 1941, when 'white' bread was the staple food.

RESULTS

It will be seen from Tables 2 and 3 that the consumption of national wheatmeal was followed, under the conditions of our experiment, by an

Table 2. *Data relating to the calcium balance of six volunteers*

Volunteer no.	Week ending	Diet	Intake of Bread (g./week)	Intake of Milk (ml./week)	Ca in food (g./week)	Ca in excreta (g./week) Urine	Faeces	Total	Ca balance (g./week)	Average daily Ca Intake (g.)	Balance (g.)
1	2. ix. 41	Wheatmeal bread	6160	600	4·21	1·56	3·52	5·08	−0·87		
	9. ix. 41	,,	7080	600	3·93	1·61	3·46	5·07	−1·14	0·580	−0·143
	16. ix. 41	,,	7140	700	4·26	1·53	3·68	5·21	−0·95		
	23. ix. 41	,,	6260	600	3·82	1·60	3·27	4·87	−1·05		
	14. x. 41	White bread	5850	950	3·52	1·71	2·37	4·08	−0·56		
	21. x. 41	,,	5770	1100	4·61	1·73	3·08	4·81	−0·20	0·625	−0·022
	28. x. 41	,,	5070	1400	4·62	1·67	3·02	4·69	−0·07		
	4. xi. 41	,,	5450	1400	4·74	1·51	3·02	4·53	+0·21		
2	29. viii. 41	Wheatmeal bread	5800	600	3·92	1·52	4·21	5·73	−1·81		
	5. ix. 41	,,	4990	700	3·58	1·59	2·66	4·25	−0·67	0·531	−0·141
	12. ix. 41	,,	5620	700	3·68	1·70	2·69	4·39	−0·71		
	19. ix. 41	,,	5140	700	3·72	2·03	2·43	4·46	−0·74		
	17. x. 41	White bread	5050	1050	3·30	1·98	1·75	3·73	−0·43		
	24. x. 41	,,	3930	1400	3·67	1·63	2·42	4·05	−0·38	0·504	−0·054
	31. x. 41	,,	4860	1400	3·63	1·64	2·23	3·87	−0·24		
	7. xi. 41	,,	5600	1400	3·52	2·09	1·90	3·99	−0·47		
3	28. viii. 41	Wheatmeal bread	5108	700	4·13	2·05	2·94	4·99	−0·86		
	5. ix. 41	,,	5150	700	4·02	2·35	2·83	5·18	−1·17	0·622	−0·073
	12. ix. 41	,,	5270	700	4·46	2·17	2·29	4·46	±0		
	19. ix. 41	,,	5301	700	4·80	2·46	2·36	4·82	−0·02		
	3. x. 41	White bread	6387	600	3·34	2·27	1·49	3·76	−0·42		
	10. x. 41	,,	5813	500	3·65	2·05	1·38	3·43	+0·22	0·574	+0·038
	17. x. 41	,,	5494	1050	4·40	2·44	1·62	4·06	+0·34		
	24. x. 41	,,	5584	1400	4·67	2·16	1·58	3·74	+0·93		
4	29. viii. 41	Wheatmeal bread	5368	700	4·42	1·86	2·51	4·37	+0·05		
	5. ix. 41	,,	5178	700	3·67	2·14	2·45	4·59	−0·92	0·560	−0·120
	12. ix. 41	,,	5165	700	4·13	2·11	3·03	5·14	−1·01		
	19. ix. 41	,,	4734	600	3·44	2·05	2·86	4·91	−1·47		
	17. x. 41	White bread	4108	1050	3·40	1·85	2·74	4·59	−1·19		
	24. x. 41	,,	4695	1400	4·40	2·18	2·44	4·62	−0·22	0·564	−0·038
	31. x. 41	,,	4305	1400	4·05	1·59	1·80	3·39	+0·66		
	7. xi. 41	,,	4518	1400	3·96	1·60	2·67	4·27	−0·31		
5	2. ix. 41	Wheatmeal bread	4980	700	3·86	1·77	2·46	4·23	−0·37		
	9. ix. 41	,,	5360	700	3·85	1·92	2·80	4·72	−0·87	0·516	−0·118
	16. ix. 41	,,	5240	600	3·62	1·91	2·61	4·52	−0·90		
	23. ix. 41	,,	4560	700	3·10	1·79	2·47	4·26	−1·16		
	21. x. 41	White bread	4710	1250	3·81	1·63	2·77	4·40	−0·59		
	28. x. 41	,,	4730	1400	4·30	1·82	2·78	4·60	−0·30	0·600	−0·033
	4. xi. 41	,,	5000	1400	4·48	1·61	2·67	4·28	+0·2		
6	2. ix. 41	Wheatmeal bread	5121	700	3·60	1·58	2·22	3·80	−0·20		
	9. ix. 41	,,	5068	700	3·85	1·76	2·37	4·13	−0·28	0·491	−0·065
	16. ix. 41	,,	4400	700	3·34	1·71	2·11	3·82	−0·48		
	23. ix. 41	,,	4512	700	2·96	1·74	2·07	3·81	−0·85		
	7. x. 41	White bread	3791	700	2·34	1·41	0·97	2·38	−0·04		
	14. x. 41	,,	4667	1350	3·10	2·30	1·17	3·37	−0·27	0·442	−0·027
	21. x. 41	,,	4192	1400	3·27	2·00	2·20	4·20	−0·93		
	28. x. 41	,,	4306	1400	3·65	1·85	1·33	3·18	+0·47		

average Ca loss of 0·110 g./day. When wheatmeal was replaced by 'white' flour the average balance was still slightly negative, viz. 0·023 g., but this is less than one-quarter of the loss during the wheatmeal period. The effect of wheatmeal is clear-cut in every individual case.

These results are in accordance with those of McCance & Widdowson [1942]. From Table 17 of their paper we calculate the following figures:

Milling standard %	Average daily Ca intake g.	Average daily Ca balance g.
92	0·558	−0·086
69	0·512	−0·018

Table 3. *Effect of national wheatmeal on the average calcium intakes and balances of six men*

(This is a summary of Table 2; the data on national wheatmeal are the average of 24 weeks, those on white flour of 23 weeks.)

Milling standard	Average daily bread consumption (not counting approximately 80 g. flour eaten in cooked dishes) (g.)	Average daily Ca intake (g.)	Average daily Ca balance (g.)
National wheatmeal (85 % extraction)	766	0·550	—0·110
White flour (75 % extraction)	707	0·552	—0·023

In these experiments the bread consumption was somewhat lower (about 530 g./day) than in ours (about 730 g./day). This explains why, in our subjects, the Ca loss was greater than in those of McCance & Widdowson, although we used 85 % extraction flour whilst the data of McCance & Widdowson refer to a 92 % extraction flour.

We wish to emphasize that the conditions under which the adverse effect of national wheatmeal on the Ca balance was observed were exceptional ones. They included an average daily intake of 1·7 lb. of bread (requiring an extra jam ration to make it palatable) as well as a deliberate and severe curtailment of the Ca supply. In a preliminary experiment where the average wheatmeal bread intake was about 1 lb./day and the average daily Ca supply 0·94 g., the Ca balance was in equilibrium (Table 4).

Table 4. *Ca balance at higher level of Ca intake and lower level of wheatmeal bread consumption*

(Average of 14 weekly periods; 5 subjects.)

	g.
Average wheatmeal bread intake	456
Average daily Ca intake	0·941
Average daily Ca excretion	0·911
Average daily Ca balance	+0·030

SUMMARY

1. Six adult male volunteers were kept on a diet containing a large proportion of bread (on average 766 g. wheatmeal bread of 85 % extraction or 707 g. 'white' bread of 75 % extraction) and about 0·55 g. Ca/day. Ca was determined in food, faeces and urine.

2. In every case national wheatmeal had an adverse effect on the Ca balance. On average 0·110 g. Ca was lost daily during the wheatmeal regime whilst 0·023 g. was lost during the white flour regime.

3. The results are in accordance with those obtained by McCance & Widdowson on 69 and 92 % extraction flours, and support the present policy of adding Ca salts to national wheatmeal.

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Colorimetric Determination of Total, Free and Ester Cholesterol in Tissue Extracts

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Kelsey's method [1939] for the determination of total, free and ester cholesterol was tested in the course of analyses of lipid extracts of various tissues (liver, kidney, heart, adrenals and blood). This method seemed to be particularly useful, because not only cholesterol, but also total fatty acids, and fatty acids derived from neutral fats and cholesteryl esters, could be determined on one sample. Kelsey claimed that the cholesterol obtained by his method, and estimated colorimetrically, is pure. However, in my experience, the cholesterol thus obtained is contaminated by substances which often considerably influence the colour developed with the Liebermann-Burchard reagents, and make accurate colorimetric reading impossible. Apart from this disturbing factor, the method gave very low results: cholesterol added to lipid mixtures could not be satisfactorily recovered.

After some experiments a technique has been evolved which retains the advantages of Kelsey's method and is essentially a combination of the method of Schoenheimer & Sperry [1934], as modi-

fied by Sturges & Knudson [1938], with that of Kelsey. The cholesterol is precipitated with alcoholic digitonin in acidified medium at room temperature, and the digitonide separated by centrifuging. The decomposition of digitonide and the isolation of cholesterol are done by Kelsey's method, i.e. by boiling with benzene and precipitating the digitonin by light petroleum. There was no interference with the colour developed by the Liebermann-Burchard reaction with cholesterol samples obtained in this way; the colour was indistinguishable from that of the standard when compared in a colorimeter.

Procedure

Total cholesterol. Samples of ethanol-ether extracts of tissues containing about 0·5 mg. of cholesterol are evaporated almost to dryness in a conical flask on a hot plate, then 10 ml. of ethanol-ether (1:1, v/v) and 0·2 ml. of a solution of KOH in H_2O containing 40 g./100 ml. of solution are added. The mixture is brought to boiling, the flask loosely stoppered and placed in an oven at 75° for 1·5–2 hr. After this time a few drops of phenol red are added and the mixture acidified with HCl of such a strength that 1 ml. makes the mixture definitely acid. After acidification the contents of the flask are evaporated almost to dryness on a hot plate, the last traces of ethanol being blown out by a current of air. The salt in the residue is dissolved in 0·5–1 ml. of water. If more water is added, it is likely that the extraction will be incomplete. The flask is replaced on the hot plate and, when the mixture is hot, 10 ml. of light petroleum (L.P.) (b.p. 40–60°) are poured into the flask, which is vigorously rotated. After a few minutes' standing, the L.P. extract is decanted from the coloured fluid into a 15 ml. conical centrifuge tube and concentrated, with the aid of a boiling rod,* by placing the tube in a beaker of hot water (about 50°), in order to allow space for the following extracts. The contents of the conical flask are extracted three times more with boiling L.P. After the last extraction the mouth of the flask is also rinsed with a few drops of L.P. All the extracts are combined in the centrifuge tube, and finally concentrated to approximately 0·5 ml. [Kelsey, 1939].

Cholesterol is precipitated by addition of 4 drops of 5% (w/w) HCl and of 4 ml. of a 0·25% (w/v) solution of digitonin in ethanol. The boiling rods are left in the tubes and used for thorough mixing of the contents. The tubes are left standing overnight in a sealed jar at room temperature. The boiling rods are removed to a numbered rack and the cholesterol digitonide separated by centrifuging for 5 min. The supernatant fluid (fluid A) is decanted and can be saved for the determination of total fatty acids. The cholesterol digitonide and the sides of the tube are washed with 3 ml. of acetone-ether (1:2, v/v), the digitonide well stirred with the boiling rod and again centrifuged for 5 min. The washing is decanted and the digitonide washed twice more with 3 ml. portions of ether [Schoenheimer & Sperry, 1934; Sturges & Knudson, 1938].

To the digitonide thus purified 7 ml. of benzene are added and the boiling rod replaced. The tube is put into a beaker of hot water (80–82°) and the benzene kept boiling for 45 min.; the amount of benzene is kept above 5 ml. by adding small volumes of benzene to the tube from time to time. After 45 min. the benzene is concentrated to 2 ml., and while still hot, 9 ml. of L.P. are rapidly added to precipitate digitonin. Thorough mixing is essential, and about 10 min. are allowed for the precipitation of digitonin. After centrifuging for 5 min. the supernatant fluid, which contains the cholesterol, together with three successive L.P. washings of the digitonin, is poured into a small glass-stoppered conical flask and evaporated to dryness on a hot plate, the last traces of L.P. being blown out [Kelsey, 1939].

When the flasks are cool, the cholesterol is dissolved in 5 ml. of $CHCl_3$, and the flasks placed in a dish containing water kept at 18°. After a few minutes, 2 ml. of a freshly prepared mixture of acetic anhydride and conc. H_2SO_4 (20:1, v/v), also cooled to 18°, are added and well mixed with the $CHCl_3$. The flasks are kept in H_2O and placed in a dark place. Standards are prepared at the same time, and after 60 min. the colour comparison is made in a colorimeter [cf. Ireland, 1941]. Only the samples which are actually to be compared are removed from the darkness.

If the amount of cholesterol in the sample is over 1 mg., more digitonin can be used than described. In this case 14 ml. of benzene are used for the decomposition of digitonide, and at the stage of colour development the cholesterol is dissolved in 10 or 15 ml. of $CHCl_3$ and 4 or 6 ml. of the acetic anhydride-H_2SO_4 mixture are added.

If total fatty acids are to be determined, the supernatant fluid (fluid A) from the cholesterol digitonide is decanted into another centrifuge tube and concentrated to about 1 ml.; the acetone-ether washing of the digitonide is added and the whole again concentrated to 1 ml. The ether washings are also added; excess digitonin will be precipitated and is centrifuged off. The supernatant fluid contains the fatty acids. This fluid is quantitatively decanted and evaporated to dryness and the fatty acids dissolved in a small volume of L.P. Their amount can be determined by the dichromate oxidation method of Bloor [1928]. The fatty acids thus isolated cannot be used for the determination of their iodine values because, apparently during the treatment with digitonin and HCl, they become more saturated.

Free cholesterol. To determine free cholesterol, it is necessary to separate first the phospholipids from a suitable sample of the ethanol-ether extract of the tissues, since digitonin will also precipitate the phospholipids [McEwen & MacLachlan, 1941]. A sample of the extract is evaporated to dryness on a hot plate and the residue thoroughly extracted with L.P.; the L.P. extract is concentrated to 2 ml. in a conical centrifuge tube and 7 ml. of acetone and 3 drops of a saturated solution of $MgCl_2$ in ethanol are added to precipitate phospholipids [Bloor, 1929]. After centrifuging, the phospholipid-free supernatant fluid, which contains chiefly free cholesterol, cholesteryl esters and neutral fats, is decanted into another centrifuge tube. The phospholipids are washed with two 2 ml. portions of acetone. These are combined with the decantate and the whole concentrated to 0·5 ml. by immersing the centrifuge tube in a beaker of hot water (about 60°) and by using a boiling rod. 4 ml. of the digitonin solution and 4 drops of 5% HCl are added and the tubes left overnight in a sealed jar. The cholesterol digitonide is separated by centrifuging. The supernatant fluid (fluid B) is decanted and can be saved for the determination of ester cholesterol, and of fatty acids derived from neutral fats and cholesteryl esters. The purification, decomposition of the digitonide and the colour development are done as described for total cholesterol.

* A 1 cm. long capillary tube fused on to a 2 mm. glass rod.

Ester cholesterol. Fluid B, supernatant after the free cholesterol has been separated as digitonide, contains cholesteryl esters, neutral fats and excess digitonin. After the excess digitonin has been precipitated as indicated for the determination of total fatty acids (treatment of fluid A), the supernatant fluid is decanted, evaporated and saponified, extracted with L.P., and the cholesterol determined by precipitation with digitonin as described for total cholesterol. If desired, the fatty acids derived from neutral fats and cholesteryl esters can also be separated in the same way as total fatty acids.

Table 1 shows the amounts of cholesterol recovered after it has been added, dissolved in $CHCl_3$, to ethanol-ether extracts of known cholesterol content. The accuracy of the method is further illustrated by the results given in Table 2, which are examples of determinations of total, free and ester cholesterol on various tissue extracts.

Table 1. *Recovery of cholesterol added in $CHCl_3$ solution to ethanol-ether extracts of tissues*

Cholesterol added (mg.)	Cholesterol recovered (mg.)	Cholesterol added (mg.)	Cholesterol recovered (mg.)
0·200	0·199	0·500	0·496
0·200	0·199	0·500	0·502
0·200	0·198	0·500	0·498
0·200	0·200	0·500	0·500
0·400	0·400	1·000	1·000
0·400	0·400	1·000	1·000

Table 2. *Results of total, free and ester cholesterol determinations in extracts of various tissues*

Material	Cholesterol content mg./100 g. moist tissue: Total	Free	Ester
Human kidney cortex	220	202	22
	224	198	27
	381	358	23
	326	219	107
Human kidney medulla	193	137	45
	230	171	60
	255	194	64
Human liver	252	225	30
Human adrenal	2500	420	2100
Human plasma	152*	65*	85*
Human heart	88	74	12

* mg./100 ml. of plasma.

SUMMARY

A method is described for determination of total, free and ester cholesterol in tissue extracts by a combination of the method of Schoenheimer and Sperry (modified by Sturges and Knudson) with that of Kelsey.

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Adrenaline and Related Substances in Blood and Tissues

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The results obtained with the colorimetric method of Shaw [1938], for the assay of adrenaline in blood and tissues, cannot be attributed to adrenaline alone, since the denominator of the 'specific ratio', i.e. the ratio of the results with acid and with alkali [cf. Shaw, 1938], was found, with a few exceptions, to be considerably lower than that of adrenaline itself (Table 1). Quantitative adsorption of ascorbic acid by $Al(OH)_3$ at a *p*H of about 8·5 has been regularly observed by the writer, and thus ascorbic acid must be regarded as participating in the colorimetric results, contrary to a statement by Shaw [1938]. Despite the low value of the denominator of the specific ratio (henceforth designated d.s.r.) for ascorbic acid (0·3), this substance alone cannot be responsible for the low d.s.r.'s generally found in blood and most tissues, including the adrenal glands (Table 1). Its chromogenic intensity is too weak (Table 2) and its reported average concentrations in blood and tissues (Table 3) are too small.

Many chromogenic substances other than adrenaline and ascorbic acid are eliminated by the adsorption procedures of the method. Certain chromogenic hormonal steroids, which are by themselves not adsorbed on $Al(OH)_3$, were originally believed [Raab, 1941 *a*, *b*, *c*] to be partially adsorbed at *p*H 8·5 in the presence of adrenaline, owing to the formation of a hypothetical 'adreno-cortical' compound. This conception is now abandoned since it was found that steroids pass quantitatively into

Table 1. *Normal averages of colorimetric results obtained from tissues and blood*

Organ	Content of adsorbable chromogen (colour units/g. or ml.)	Denominator of the specific ratio*	No. of specimens
Adrenal glands			
(rat)	259,311	1·26	56
(man, medulla)	232,000	1·33	6
(man, cortex)	23,820	1·51	6
Spleen (rat)	2,322	1·00	20
Testis (rat)	1,960	1·07	2
Brain (rat)	1,775	0·89	19
Liver (rat)	1,765	0·98	26
Kidney (rat)	1,180	1·08	28
(man)	1,438	1·32	14
Heart (rat)	946	1·00	40
(man)	698	1·37	21
Muscle (rat)	898	1·88	19
Aorta (man)	493	3·50	17
Renal artery (man)	258	3·55	8
Blood (man)	156	1·06	51
(dog)	168	?	3
(cat)	123	?	2

* A value greater than 2·0 indicates pure or almost pure adrenaline, or sympathin; a d.s.r. between 2·0 and 1·0 indicates the presence of both adrenaline and other adsorbable chromogens with a d.s.r. lower than that of adrenaline; a d.s.r. of 1·0 or below indicates the exclusive presence of adsorbable chromogens other than adrenaline or sympathin.

Table 2. *Chromogenic properties and adsorbability of some substances related to adrenaline*

Substance	Colour intensity compared with that of adrenaline	Denominator of the specific ratio	Adsorbability at *p*H 8·5
Adrenaline	1	2·0–3·5*	+
Ascorbic acid	1/320	0·3	+
Adrenalone	1/11	1·1	+
Dihydroxyphenylalanine	1/3	1·1	+
Epinine (synthetic)	1/6	1·1	+
Leukoadrenochrome (?)	?	1·0	+
Pyrocatechol	1/9	0·6	+
Adrenochrome	1/5	1·0	–
Benzoquinone	1/10	2·4	–
Quinol	1/20	2·7	–

* These d.s.r.'s are found if adrenaline is subjected to the adsorption procedures; without these it is about 5·0 [cf. Shaw, 1938].

Table 3

The average amounts of ascorbic acid present in various tissues according to data collected from the literature would have to be multiplied by the figures given below in order to lower the d.s.r. of pure adrenaline to the d.s.r.'s actually found in the following tissues:

Adrenal glands	10–20	Heart muscle	7
Spleen	2	Striated muscle	1–1½
Liver	2	Brain	3–4
Kidney	3–4	Blood	4–5

organic solvents (chloroform, petroleum ether) from aqueous solutions containing adrenaline, while the latter remains in the water. The original erroneous conception had been based on the following facts: (*a*) increased colour effect and lowered d.s.r. of adrenaline solutions to which cortical and other steroids had been added; (*b*) quantitative recovery of the entire chromogenic material in chloroform extracts of dried blood and tissues. The first phenomenon is probably to be explained by an alteration of the adrenaline molecule, rather than by a combination with the added steroids; the second one seems to be due to the fact pointed out by Kendall [1942] and confirmed by the writer, that adrenaline, which is otherwise chloroform-insoluble, becomes chloroform-soluble in the presence of lecithin. The same was found to be true also for ascorbic acid and adrenalone.

If a chromogenic substance is to be estimated by Shaw's method, it must be adsorbed by $Al(OH)_3$ at a *p*H of about 8·5. A search for adsorbable substances which might account for the low d.s.r.'s found in blood and tissues revealed the fact that this condition was fulfilled only by such chromogenic substances as possessed two or more adjacent OH groups on a benzene ring or heterocyclic ring containing oxygen. Thus, besides adrenaline proper and ascorbic acid, a number of adrenaline-like substances with an intact catechol nucleus were found to be detectable with the method used (Table 2). Pyrogallol and gallic acid which, by virtue of their adjacent hydroxyl groups, are also adsorbable, are probably not present in blood and tissues.

The integrity of the catechol nucleus of adrenaline and related substances appears to be of fundamental significance for the adsorbability by $Al(OH)_3$ in an alkaline medium, as well as for the especially high electrical potential of adrenaline and related catechols [Ball & Chen, 1933] and, according to Tainter [1930] and Vaughan, Perkins & Derbes [1942], for the sympathomimetic properties of these substances. Adrenochrome and quinones give a somewhat intense colour reaction (Table 2), but, lacking OH groups in adjacent positions, they are not adsorbable by $Al(OH)_3$ at *p*H 8·5. Their electrical potential is lower than that of catechol compounds [Ball & Chen, 1933] and their pharmacodynamic effects are of a prevailingly depressant character, such as is the case with adrenoxin [Heirman & Bacq, 1940], and other quinones [Oster & Sobotka, 1942]. The conclusion is reached that, except in those instances in which a d.s.r. greater than 2·0 indicates the presence of pure or almost pure adrenaline [or sympathin; Cannon & Lissák, 1939], the results obtained with Shaw's method are due not only to adrenaline itself and to ascorbic acid, but also to certain adrenaline-like catechol compounds with a d.s.r. lower than that of adrenaline.

Even the isolated human adrenal medulla did not yield pure adrenaline. Its very high content of adsorbable chromogenic material (Table 1) had an average d.s.r. as low as 1·33. The d.s.r.'s of rat adrenals were similarly low and showed only insignificant alterations under various experimental conditions.

The colorimetric results of Shaw's method as applied by Raab [1941 *a*, *b*] are expressed in 'colour units', each unit corresponding to the chromogenic intensity of 0·001 μg. of adrenaline.

The adrenal glands contain by far the largest amounts of adsorbable chromogen (Table 1). Its concentration in the cortex of human glands was about one-tenth of that in the medulla, possibly due in part to post-mortem contamination.

Relatively large amounts of an adsorbable chromogen not identical with adrenaline itself were also present in liver, kidney and brain (Table 1), organs which are known to remove adrenaline most efficiently from perfusing fluids [Bernheim, 1942] and to contain the highest concentrations of an amine-oxidase which destroys adrenaline through oxidative deamination of its side-chain [Blaschko, Richter & Schlossmann, 1937]. The striated muscle, on the other hand, in which the adrenaline-destroying oxidase is not present [Blaschko *et al.* 1937] contained mainly adrenaline, and the proportion was even higher in the tissue of arterial walls. Since muscular tissue has only a weak tendency to remove adrenaline from perfusing fluids [Pak, 1926], it seems probable that at least part of its content in chromogenic material with a high d.s.r. is identical with sympathin deriving from peripheral adrenergic neurones [Cannon & Lissák, 1939].

The lowest content of adsorbable chromogen was found in blood with a d.s.r. close to 1·0 under standard conditions, which indicates that adrenaline itself, if it is present in blood, circulates in very small amounts only. Bloor & Bullen [1941], using another modification of Shaw's method, came to the same conclusion.

The doubtful specificity of Shaw's method made it desirable to investigate its usefulness as a criterion of adrenal activity by correlating its results with a variety of experimental and clinical conditions which are known or suspected to be accompanied by alterations of adrenal secretion or hormone deposition.

More than 3000 determinations were carried out for this purpose with the following results, some of which have been published in detail [Raab, 1941 *a*, *b*, *c*, *d*, *e*, 1942]. The term 'chromogen' signifies material which is adsorbed by $Al(OH)_3$ at a *p*H of about 8·5 and estimable by Shaw's method.

(1) *Injection of adrenaline* (0·5–1·0 mg.) was followed within 30 min. by an average increase of the d.s.r. of human blood from 1·11 to 1·58, while the total level of chromogen showed only an insignificant elevation (10 exp.). In rats, both the concentration of chromogen and the d.s.r. were increased in the kidney, liver, brain and particularly in the heart muscle (+138 %). The latter displayed an outstanding tendency to accumulate both injected adrenaline and other catechol compounds (adrenalone, dihydroxyphenylalanine, pyrocatechol) (52 exp.).

(2) *Injection of thyroxin* in rats increased the myocardial d.s.r. (10 exp.). After pretreatment with thyroxin the myocardial accumulation of injected adrenaline was intensely accelerated and increased with resulting cardiac death within a few minutes (6 exp.).

(3) *Injection of desoxycorticosterone acetate* in man was followed by a slight increase of the d.s.r. of the blood (12 exp.). In rats it was followed by an increased chromogen content of kidney, liver and heart muscle with only little change of the d.s.r. (30 exp.). The vasopressor effect of adrenaline was markedly increased by pretreatment with desoxycorticosterone acetate (6 exp.).

(4) *Injection of insulin.* 10–15 units applied intravenously increased the d.s.r. of human blood from 1·09 to 1·40 within 45 min. (5 exp.). In rats the chromogen concentration and d.s.r. of the heart muscle, liver, brain and striated muscle were increased (40 exp.).

(5) *Vitamin B_1 deficiency.* The myocardial adsorbable chromogen was markedly increased in rats. Administration of thiamin chloride restored conditions to normal within a few days (15 exp.).

(6) *Exposure to cold.* Increase of chromogen and d.s.r. in the heart muscle of rats; in the striated muscle the total chromogen was diminished but the d.s.r. was increased (20 exp.).

(7) *Physical exercise.* In fourteen normal persons a slight elevation of the average blood chromogen and an elevation of the d.s.r. from 0·86 to 1·28 took place within 2–5 min.; return to normal followed within 15 min. In rats a considerable increase of the myocardial chromogen and d.s.r. occurred (30 exp.) and in the striated muscle a diminution of the total chromogen accompanied by an increase of the d.s.r. (30 exp.).

(8) *Adrenalectomy* in rats was followed by a diminution of the chromogen of the heart muscle (22 %), kidney (7 %), liver (33 %), brain (24 %), striated muscle (19 %) and spleen (47 %), with only little change of the d.s.r. (60 exp.). In one adrenalectomized dog the blood chromogen level decreased steadily without, however, falling below the lowest limit of normal.

(9) *Essential hypertension.* At rest a normal blood chromogen level in 36 cases, but an increased average elevation of the blood chromogen after exercise (14 exp.).

(10) *Angina pectoris.* In twelve cases a normal chromogen level at rest, but abnormally high elevations of the blood chromogen after exercise (average from 140 to 195 colour units/ml.).

(11) *X-ray irradiation of the adrenal region* diminished the myocardial chromogen of rats after exercise (14 exp.). In seven patients with angina pectoris the abnormal chromogen discharges were abolished together with the anginal symptoms for periods of several months.

(12) *Pulmonary œdema* in two cardiac patients was accompanied by an abnormal increase of the blood chromogen level.

(13) *Kidney insufficiency* with uræmia was regularly accompanied by a strikingly elevated chromogen level of the blood (average 372 colour units/ml.) with an average d.s.r. of 1·13, probably due to impaired excretion (17 cases).

(14) *Cushing's disease.* Four cases showed a blood chromogen level near the upper limit of the normal range (average 215 colour units/ml.).

(15) *Addison's disease.* In one mild case the chromogen level was moderately below the normal average (98 colour units/ml.).

(16) '*Hypertensive*' *and* '*arteriosclerotic*' *heart disease with cardiac failure.* In the heart muscle either the total chromogen or the concentration of adrenaline proper was abnormally high in 78% of 32 cases. In one case the myocardial chromogen was abnormally low after therapeutic sympathectomy, which may be comparable with the disappearance of chromogenic material from the cat's heart after denervation, as observed by Cannon & Lissák [1939].

(17) *Unexplained sudden cardiac death* of a young athlete. The chromogen concentration of the heart muscle was excessively high (1622 colour units/g. against an average of 698 in 21 normal hearts).

(18) *Ageing and arteriosclerosis.* The average chromogen concentrations of heart, kidneys and arterial walls were found to be lowest in early childhood. They increased gradually and reached a maximum in senile age. Particularly high chromogen concentrations were found in arteriosclerosis (138 specimens).

(19) *Adrenal tumours.* In two cases with adrenal tumours, abnormally high chromogen concentrations were found in arterial walls and kidneys.

SUMMARY

1. The results obtained with the method of Shaw [1938] for the assay of 'adrenaline' in blood and tissues (adrenal glands, liver, spleen, kidney, brain, heart) are due to the presence in these tissues not only of adrenaline itself, but also of adrenaline-like substances which contain a catechol nucleus, and of ascorbic acid. Thus, although the method is not specific for adrenaline, it can be used for investigations of adrenal and adrenergic function in a broader sense.

2. The results of numerous determinations carried out under various experimental and clinical conditions are in general accord with existing knowledge or concepts regarding adrenal and adrenergic physiology and pathology.

3. Contrary to the author's earlier view, it is no longer held that the low denominator of the 'specific ratio' obtained by Shaw's method for many tissues is due to the presence of adrenaline in combination with cortical hormones. This ratio is low when adrenaline-like compounds or ascorbic acid are present.

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Studies on Cholinesterase

3. SPECIFIC TESTS FOR TRUE CHOLINESTERASE AND PSEUDO-CHOLINESTERASE

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In the animal body two esterases capable of hydrolysing acetylcholine exist: a true cholinesterase, acting exclusively on certain choline esters, and a non-specific enzyme, which also hydrolyses a variety of non-choline esters [Mendel & Rudney, 1943]. The non-specific or pseudo-cholinesterase, purified from horse serum and from dog pancreas [Mendel & Mundell, 1943], exhibits its maximum activity at high concentrations of acetylcholine (above 300 mg./100 ml.), and is far less active at low substrate concentrations; the specific or true cholinesterase, on the other hand, found in red blood cells and brain tissue, displays its greatest activity at low substrate concentrations (below 3 mg./100 ml.) and is strongly inhibited at high concentrations of acetylcholine.

These differences in activity make it possible to decide whether an enzyme solution contains mainly pseudo- or true cholinesterase. The activity is measured towards 0·06*M* and 0·0006*M* acetylcholine: a ratio (activity at 0·06*M*/activity at 0·0006*M* acetylcholine) of 3, as found with horse serum, indicates a preponderance of pseudo-cholinesterase; conversely, a ratio of 0·3, obtained, for example, with erythrocytes, points to the predominance of true cholinesterase.

To measure quantitatively the activities of true and pseudo-cholinesterase, new tests based on the following facts are used:

(1) Acetyl-β-methylcholine (*dl*-acetyl-β-methylcholine chloride—'Mecholyl'—Merck) is hydrolysed by true but not by pseudo-cholinesterase.

(2) Benzoylcholine is hydrolysed by pseudo- but not by true cholinesterase. The benzoylcholine chloride used was prepared according to Fourneau & Page [1914] and Baer [1942], and was further purified by refluxing with absolute acetone. Both preparations had m.p. 206°.

(3) Neither acetyl-β-methylcholine nor benzoylcholine is hydrolysed by the common esterase, an enzyme capable of hydrolysing glycerides and aliphatic esters [Easson & Stedman, 1937].

EXPERIMENTAL

(*a*) *Specificity of substrate.* Table 1 gives the results of experiments in which preparations of true and pseudo-cholinesterase were allowed to act on acetyl-β-methylcholine and on benzoylcholine. All measurements of enzyme activity were carried out manometrically, as previously described [Mendel & Rudney, 1943]. These results show that acetyl-β-methylcholine is hydrolysed very slowly, if at all, by pseudo-, but readily by true cholinesterase. Benzoylcholine, on the other hand, is not hydrolysed at all by true cholinesterase, but fairly rapidly by pseudo-cholinesterase. Fortunately, neither of these two esters is hydrolysed by the common esterase. When guinea-pig plasma, which contains both cholinesterases as well as common esterase, is allowed to act on benzoylcholine or acetyl-β-methylcholine in the presence of $10^{-5}M$ eserine (a concentration completely inhibiting both cholinesterases, but not interfering with the action of the common esterase), no hydrolysis of either acetyl-β-methylcholine or benzoylcholine occurs (Table 2).

Table 1. *Hydrolysis of acetyl-β-methylcholine and benzoylcholine*

Type of 'cholinesterase'	Hydrolysis (μl. CO_2 evolved in 20 min.) of: Acetylcholine 0·02*M*	Acetyl-β-methylcholine 0·03*M*	Benzoylcholine 0·006*M*
Pseudo-cholinesterase:			
Horse serum, purified preparation (Q_{Ach} 52,000)	162	1	41
Dog pancreas, purified preparation (Q_{Ach} 430,000)	148	1	37
True cholinesterase:			
Human red blood cells, purified preparation	90	76	0
Dog brain (*nucleus caudatus*)	99	79	0

All the results given above and in subsequent tables are corrected for non-enzymic hydrolysis.

Table 2. *Effect of eserine on the hydrolysis of various substrates by guinea-pig plasma*

Conc. of eserine *M*	Hydrolysis (μl. CO_2 evolved in 10 min.) of: Tributyrin 0·003*M*	Acetylcholine 0·06*M*	Acetyl-β-methylcholine 0·03*M*	Benzoylcholine 0·006*M*
0	1560	321	28	118
10^{-5}	1510	0	0	0

All results represent the activity of 0·5 ml. plasma.

(*b*) *Estimation of true and pseudo-cholinesterase in blood.* Tables 3 and 4 show that enzyme concentration and reaction velocity are directly proportional under the experimental conditions selected.

Table 3. *Hydrolysis of acetyl-β-methylcholine (0·03*M)

Haemolysed human erythrocytes (ml.)	μl. CO_2/10 min.
0·05	7·0
0·1	13·8
0·2	28·2
0·3	42·0

Table 4. *Hydrolysis of benzoylcholine (0·006*M)

Horse plasma (ml.)	μl. CO_2/10 min.
0·05	12·8
0·1	26·0
0·2	52·6
0·3	76·8

On the basis of the observations of Tables 1–4, quantitative estimations of true and of pseudo-cholinesterase in blood were made with acetyl-β-methylcholine and benzoylcholine respectively. For comparison, the activity of the blood was also measured with the old method, with acetylcholine (0·06*M* and 0·0006*M*) as substrate. The procedure adopted was as follows:

Freshly drawn oxalated blood was used, and care was taken to avoid haemolysis. The blood was centrifuged for 15 min. in a haematocrit at 2000 r.p.m. The blood cells were washed twice with 0·9% NaCl and then brought to

the original blood volume with water. 0·2 ml. of this solution, in a total volume of 3 ml., was used for the estimation of blood-cell activity with acetyl-β-methylcholine and benzoylcholine. For the measurement of the pseudo-cholinesterase in plasma, 0·1–0·2 ml. of plasma, in a total volume of 3 ml., proved sufficient, while 1 ml. of plasma, in a total volume of 6 ml., was required to measure the activity of the true cholinesterase, since the plasma of several species contains only small amounts of this enzyme. 0·03 *M* acetyl-β-methylcholine and 0·006 *M* benzoylcholine

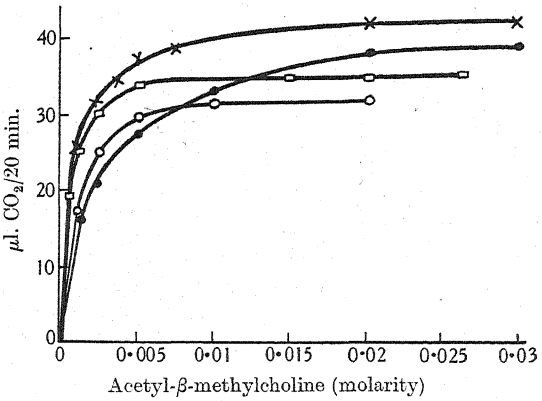

Fig. 1.

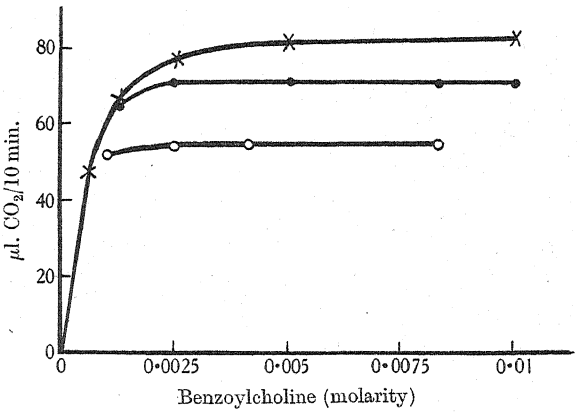

Fig. 2.

Fig. 1. Hydrolysis of acetyl-β-methylcholine. ×—× dog plasma; •—• human plasma; □—□ purified human blood cells; o—o horse plasma.

Fig. 2. Hydrolysis of benzoylcholine. ×—× dog plasma; •—• human plasma; o—o horse plasma.

were used in all experiments. These substrate levels were well above the optimum found for any one species. Some of the activity-substrate concentration curves obtained with plasma and cells are shown in Figs. 1 and 2.

RESULTS

Table 5 shows that wide variations in the activity of the true cholinesterase in plasma remain unnoticed when acetylcholine is the substrate. Since

Table 5. *Cholinesterase activity of 1 ml. dog plasma*

(μl. CO_2 evolved in 20 min.)

	Acetyl-β-methylcholine 0·03 *M*	Benzoylcholine 0·006 *M*	Acetylcholine (*a*) 0·06 *M*	Acetylcholine (*b*) 0·0006 *M*	Ratio (*a*)/(*b*)
Dog I	112	376	682	476	1·4
Dog II	54	803	1438	577	2·5

plasma II is more active than plasma I both at high and at low concentrations of acetylcholine, it would appear that plasma II contains larger amounts of both pseudo- and true cholinesterase. The presence of more pseudo-cholinesterase in plasma II is confirmed by the measurements with benzoylcholine. Tests with acetyl-β-methylcholine, however, disclose the unexpected fact that plasma II, in spite of its greater activity at low concentrations of acetylcholine, contains far smaller amounts of true cholinesterase than plasma I. The apparent discrepancies in these results are easily understood when the action of the pseudo-cholinesterase, present in greater amounts in plasma II than in plasma I, is taken into account: the pseudo-cholinesterase, being capable of exerting some effect even at low concentrations of acetylcholine, adds its activity at this substrate level to that of the true cholinesterase, and thereby renders estimations of the true cholinesterase illusory.

The experiments in Table 6 show the distribution of the two enzymes in plasma and cells of various species and disclose the following facts: (1) The blood of some species (ox, sheep) contains no pseudo-cholinesterase (Exps. 6, 7). (2) True cholinesterase is present in the blood of all species investigated (Exps. 1–11). (3) In most species the true cholinesterase is located mainly in the red blood cells, the plasma containing smaller amounts of the enzyme (Exps. 1–7). (4) Scarcity or lack of true cholinesterase in the blood cells of certain species (cats, birds) is compensated for by the presence of a comparatively larger amount of true cholinesterase in the plasma (Exps. 8–11).

The constant presence of true cholinesterase in blood suggests that this enzyme performs an essential function in the blood stream. By virtue of its ability to deal with low concentrations of acetylcholine, the true cholinesterase in all probability plays a major part in regulating the amount of acetylcholine in the circulation. Pseudo-cholinesterase, being absent from the blood of certain

Table 6. *Activity of plasma and cells contained in 1 ml. of blood*

		μl. CO_2 evolved in 20 min.						
		Acetyl-β-methylcholine 0·03 *M*		Benzoylcholine 0·006 *M*		Acetylcholine (*a*) 0·06 *M*	Acetylcholine (*b*) 0·0006 *M*	
Exp.	Source of blood	Plasma	Cells	Plasma	Cells	Plasma	Plasma	Ratio (*a*)/(*b*)
1	Man: B.M. ♂	17	848	411	0	1116	472	2·4
	H.R. ♂	16	769	409	0	1075	414	2·6
	D.M. ♀	11	761	288	0	805	288	2·8
	D.M. 2 months later	12	—	289	—	801	275	2·9
2	Horse: (i) ♂	24	169	261	0	833	241	3·5
	(ii) ♀	15	160	231	0	844	227	3·7
3	Dog: (i) ♀	37	150	224	0	409	234	1·7
	(ii) ♂	33	92	485	0	868	348	2·5
4	Guinea-pig: (i) ♂	49	224	287	0	635	336	1·9
	(ii) ♂	57	190	293	0	773	502	1·5
5	Rabbit	37	67	48	0	102	64	1·6
6	Ox	Trace	425	0	0	Trace	Trace	—
7	Sheep	31	149	0	0	32	94	0·3
8	Cat: (i) ♂	54	32	102	0	404	242	1·7
	(ii) ♀	27	16	83	0	365	183	2·0
9	Chicken	179	Trace	28	0	416	279	1·5
10	Duck	26	Trace	15	0	108	101	1·1
11	Pigeon: (i) ♀	37	0	—	—	503	361	1·4
	(ii) ♂	43	0	—	—	582	422	1·4

species, is apparently not indispensable in this respect; moreover, it could play only a minor role in controlling the level of acetylcholine in the blood, since it is unable to hydrolyse low concentrations of this substrate efficiently.

SUMMARY

1. Measurements of cholinesterase activity in which acetylcholine is used as substrate give an inaccurate picture of the levels of true cholinesterase and pseudo-cholinesterase in any mixture of the two enzymes.

2. Methods for quantitative estimations of true cholinesterase and pseudo-cholinesterase based on the use of acetyl-β-methylcholine and benzoylcholine as substrates are described.

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Bacterial Reduction of Tetrathionate

(A REPORT TO THE MEDICAL RESEARCH COUNCIL)

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(*Received 27 April 1943*)

We have previously reported that certain intestinal organisms have the power of reducing tetrathionate quantitatively to thiosulphate [Pollock, Knox & Gell, 1942]. The present paper gives the experimental evidence for this reduction and discusses some of the problems which it has suggested.

METHODS

The reaction is followed by direct iodometric titration—a method applicable to the study either of a culture growing in a tetrathionate medium, or of a washed suspension of organisms. Our work has been done mainly with washed suspensions of *Bacterium paratyphosum* B. At first these were prepared from the growth on plain nutrient agar, but the reaction then showed a long latent period before rapid reduction started, making the results difficult to analyse. When, however, the organisms were grown on agar containing a suitable concentration of tetrathionate or when a suspension prepared from the growth on plain agar was incubated with tetrathionate and mannitol or glucose for a few hours, the resulting suspension, even though repeatedly washed, was highly active, and reduction of tetrathionate was rapid and approximately linear. The significance of this difference between plain agar suspensions and tetrathionate-treated suspensions will be dealt with in a later paper.

Preparation of bacterial suspensions. 1 ml. of an 18 hr. broth culture of *Bact. paratyphosum* B was used to inoculate each of a series of flat bottles containing about 40 ml. of 2% nutrient agar prepared from tryptic digest broth. After incubation at 37° for 18 hr., the growth was washed off with 6 ml. of $\frac{1}{4}$ strength Ringer's solution. The pooled suspensions were then centrifuged and the deposit resuspended in $\frac{1}{4}$ strength Ringer's solution to give a volume of about 2·5 ml. of suspension from each bottle. This suspension contained on an average about 12·5 mg. bacterial dry weight and 1·16 mg. bacterial N/ml. In a final dilution of 1 in 5 it was then incubated at 37° in a mixture containing 0·01M tetrathionate, 0·02M mannitol and 0·2M phosphate buffer (pH 7·7) until all the tetrathionate had been reduced to thiosulphate. This usually took from 2 to 3 hr. The mixture was then centrifuged and resuspended in $\frac{1}{4}$ strength Ringer's solution up to any convenient dilution.

Preparation of tetrathionate. Tetrathionate prepared in the usual way according to the equation

$$2Na_2S_2O_3 + I_2 = Na_2S_4O_6 + 2NaI, \qquad (1)$$

inevitably contains a high proportion of iodide. This has a slight but definite inhibitory effect on reduction of tetrathionate, though it does not appear to affect the reaction in any other way. We therefore generally used pure, iodide-free tetrathionate prepared by us according to the method described by Mellor [1930], although in a few experiments we used pure tetrathionate prepared by Messrs Baird and Tatlock. After drying over H_2SO_4 the tetrathionate was kept at 0° in the dark, and under these conditions, either in the solid form or as a 0·1M solution, it remained stable for many weeks.

Quantitative estimations. (1) The total value of I_2-reducing substances was estimated by titration with standard I_2 solution after acidification with CH_3COOH, using 0·5% starch solution as indicator.

(2) Polythionates, thiosulphate, sulphite and sulphide were estimated by the methods of Kurtenacker & Wollack, and Kurtenacker & Bittner, as quoted by Starkey [1935*a*].

(*a*) Tetrathionate: samples were added to excess Na_2SO_3 (2 ml. of a saturated solution) and made just alkaline to phenolphthalein. After 5 min. excess formalin was added to fix the sulphite, followed by excess CH_3COOH. The thiosulphate formed was titrated with standard I_2 and the amount of tetrathionate calculated from the equation

$$Na_2S_4O_6 + Na_2SO_3 = Na_2S_2O_3 + Na_2S_3O_6. \qquad (2)$$

(*b*) Thiosulphate: I_2 was added to convert this all into tetrathionate by equation (1), and the tetrathionate formed estimated by the excess sulphite method.

(*c*) Estimations for sulphite were made by fixation with formalin, for sulphide by precipitation with $ZnCO_3$ and filtration, and for pentathionate and trithionate by treatment with KCN and $HgCl_2$ [Starkey, 1935*a*].

(3) Bacterial N_2 was estimated by the Kjeldahl method.

(4) Dry weight of bacterial suspensions was determined by evaporation (after thorough washing with distilled H_2O) at 120° to constant weight.

Estimation of tetrathionate reduction

Unless otherwise stated the following final concentrations were used: 0·01M tetrathionate, 0·02M mannitol and 0·2M phosphate buffer (pH 7·7) containing phenol red as indicator. Mixtures containing substrates and buffer were prepared in test tubes ($\frac{3}{4}$ in. diam.) and warmed for 30 min. at 37°. After addition of the separately warmed bacterial suspension, the tubes were incubated at 37°. A control tube was set up at the same time, containing no bacterial suspension. Samples were removed at intervals and mixed immediately with an equal volume of 10% CH_3COOH, thus stopping the reaction at once and acidifying the solution for the I_2 titration. 0·5 ml. of 0·5% starch was added to each sample, which was titrated at once with standard I_2. The presence of the organisms did not interfere with accurate titration but ordinary bacteriological precautions were taken. pH was estimated colorimetrically. With 0·2M buffer and 0·01M tetrathionate the pH fell from 7·7 to about 7·5 by the time all the tetrathionate had been reduced, but this slight drop did not affect the velocity of the reaction appreciably (see Fig. 4).

RESULTS

Fig. 1 illustrates a typical experiment (done in duplicate). It can be seen that the I_2 absorbed increases linearly with time until the reaction is complete. That this increase in I_2 absorptive power is the result of the quantitative reduction of tetrathionate to thiosulphate is shown by the following facts, illustrated by an experiment shown in Table 1.

(1) Independent estimations, by method 2 (*b*) described above, of thiosulphate formed at each stage (column 3) show close agreement with the direct I_2 titrations (column 1). The direct I_2 titrations therefore can be taken to represent with a reasonable degree of accuracy the actual amounts of thiosulphate formed.

(2) It can be seen by comparing columns (4) and (6) (which are calculated directly from the actual estimations in columns (3) and (5) respectively) that at each stage in the reaction the molecular concentration of thiosulphate formed is approximately twice that of the tetrathionate which has disappeared.

These facts are in agreement with the following equation for the reduction of tetrathionate:

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3, \qquad (3)$$

in which two molecules of thiosulphate are formed from every one of tetrathionate. Since thiosulphuric acid in solution is highly ionized this equation could also be expressed as an oxidation of H atoms to H ions:

$$2H \rightarrow 2H^+ + 2\epsilon. \qquad (3a)$$

This is consistent with the high degree of acidity developed during tetrathionate reduction, even in the absence of glucose or mannitol, necessitating the use of high concentrations of buffer.

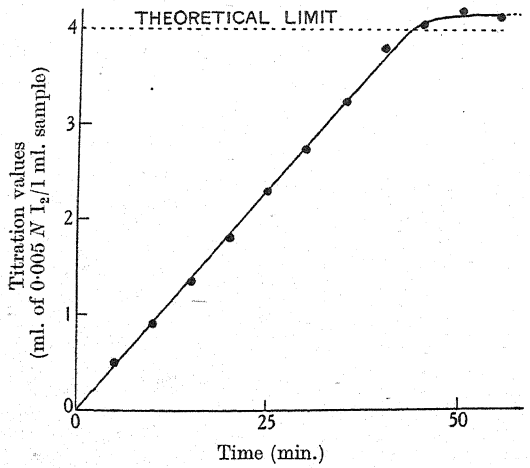

Fig. 1. Tetrathionate reduction by *Bact. paratyphosum* B. Suspension mixture contained 0·01 *M* tetrathionate, 0·2 *M* buffer and 0·02 *M* mannitol in a final volume of 12·5 ml.

The 'theoretical limit' line marks the titration value to be expected when all the tetrathionate has been converted into thiosulphate according to equation (3).

The close correspondence between the I_2 titrations and the amounts of thiosulphate estimated by method 2 (*b*) (Table 1, columns 1 and 3), is in itself evidence that not more than traces of other I_2-reducing substances are formed. It is true that if, for example, sulphite or sulphide were formed during the reaction, they would be undetectable because they would react chemically with the tetrathionate [Mellor, 1930; Mitchell & Ward, 1932]; but if this occurred to any appreciable extent the molecular ratio of thiosulphate formed to tetrathionate disappearing would inevitably be less than the figure of 2:1 which we have found experimentally. This can be seen by comparing equations (2) and (3). There was also the possibility of the formation of other polythionates (pentathionate and trithionate), but, if these were produced in any quantity, the 2:1 ratio of thiosulphate to tetrathionate would again be upset. Direct estimations of these substances (method 2 (*c*)) have failed to reveal their presence at any stage in the reaction. It is, however, possible that after complete reduction of tetrathionate small amounts of sulphite and sulphide are formed: indeed the 60 and 90 min. readings in columns (1) and (2), which show a slight decrease in the I_2 value after treatment with formalin, suggest that some traces of sulphite are present. Some further action of the organisms on the thiosulphate with evolution of H_2S is to be expected. Washed suspensions of *Bact. paratyphosum* B can certainly produce H_2S from thiosulphate, and H_2S can be readily detected after such suspensions have been incubated with tetrathionate—as soon as all the tetrathionate has been reduced to thiosulphate. Tarr [1933, 1934] has found that

Table 1. *Formation of thiosulphate and disappearance of tetrathionate in tetrathionate reduction by* Bact. paratyphosum *B*

	Titration value of 5 ml. samples with 0·01 *N* iodine		Estimation of thiosulphate formed, expressed as		Estimation of tetrathionate concentration expressed as $M \times 10^{-3}$	
Time min.	(1) Direct	(2) After treatment with formalin	(3) ml. 0·01 *M* in 5 ml. samples	(4) Conc. ($M \times 10^{-3}$)	(5) Still present	(6) Removed
			With bacterial suspension			
0	0·3	—	0	0	10·3	0
15	3·1	—	2·8	5·6	7·4	2·9
30	6·4	—	6·1	12·2	4·3	6·0
45	9·7	—	8·9	17·8	1·0	9·3
60	10·9	10·2	9·8	19·6	0	10·3
90	10·9	10·1	9·7	19·4	0	10·3
17 hr.	10·65	10·2	9·9	19·8	0	10·3
			Controls with no bacterial suspension			
0	0·15	—	—	—	10·5	0
90	0·15	—	—	—	10·7	0

The solutions, both in the control batch and in that with the bacterial suspension, were made up to a volume of 100 ml., containing 0·01 *M* tetrathionate, 0·2 *M* phosphate buffer (*p*H 7·7), and 0·02 *M* mannitol.

washed suspensions of *Proteus vulgaris* form H_2S from thiosulphate. Tarr's figures, however, showed that the rate of H_2S production was slow, while it can be seen from Table 1 that with *Bact. paratyphosum* B, even after 17 hr. incubation, there was no appreciable alteration in the amount of thiosulphate still present. This is still further evidence that in the reduction of tetrathionate to thiosulphate subsidiary reactions such as formation of sulphite or sulphide are quantitatively insignificant.

Toxicity of tetrathionate and thiosulphate

Tetrathionate, up to a concentration of $0{\cdot}05M$, has no inhibitory action on its own reduction (Table 2). It is also clear from Fig. 1, where the reaction proceeds in a straight line practically to

Table 2. *Affinity of enzyme for tetrathionate*

Initial molarity of tetrathionate	Velocity of tetrathionate reduction (absorption of I_2, as ml. of $0{\cdot}005N$ I_2, by 2 ml. samples, after 30 min. incubation)
0·05	1·3
0·02	1·3
0·01	1·35
0·004	1·35
0·002	1·35

Each tube contained washed suspension of *Bact. paratyphosum* B, $0{\cdot}2M$ buffer (*p*H 7·7), $0{\cdot}02M$ mannitol and the appropriate concentration of tetrathionate. Total vol.: 5 ml. Incubation at 37°.

completion and where the thiosulphate formed approached a concentration of $0{\cdot}02M$, that the thiosulphate likewise had no inhibitory effect within the limits used.

Hydrogen donators for tetrathionate reduction

Even in the absence of external H_2 donators, the rate of the reaction is rapid (Table 3). This residual

Table 3. *Effect of hydrogen donators on tetrathionate reduction by* Bact. paratyphosum *B*

Added H donator	Velocity of tetrathionate reduction (absorption of I_2, as ml. of $0{\cdot}005N$ I_2, by 2 ml. samples after 30 min. incubation)
Glucose	2·45
Mannitol	2·45
Lactate	1·95
Formate	2·35
No H_2 donator	1·4

Each tube was made up to contain bacterial suspension, $0{\cdot}01M$ tetrathionate, $0{\cdot}2M$ buffer (*p*H 7·7), and $0{\cdot}02M$ H_2 donator. Total vol.: 5 ml. Incubation at 37°.

or endogenous reduction constitutes a high proportion of the total reduction in the presence of mannitol or glucose. We have been unable to decrease it even by repeated washings with $\frac{1}{4}$ strength Ringer's solution. For *Bact. paratyphosum* B, glucose, mannitol, formate, and, to a less extent, lactate can act as H_2 donators for tetrathionate reduction.

Affinity of enzyme for tetrathionate

It has not been possible to estimate the rate of tetrathionate reduction with concentrations of tetrathionate lower than $0{\cdot}002M$, since the quantity of thiosulphate formed is then too small for accurate analysis. But Table 3 shows that there is no appreciable decrease in the initial velocity between $0{\cdot}05M$ and $0{\cdot}002M$, so that the affinity of the enzyme for tetrathionate must be fairly high; moreover, the reduction of tetrathionate is linear very nearly to the end of the reaction (Fig. 1). In the comparison of different velocities we have, however, confined our figures to the first half of the reaction, and not attached any significance to readings after the concentration of tetrathionate has fallen below $0{\cdot}004M$.

Some physical factors affecting tetrathionate reduction

Effect of temperature. From Fig. 2 it can be calculated that the mean Q_{10} value between 18° and 37° is 1·94 and between 37° and 43° is 2·64. At

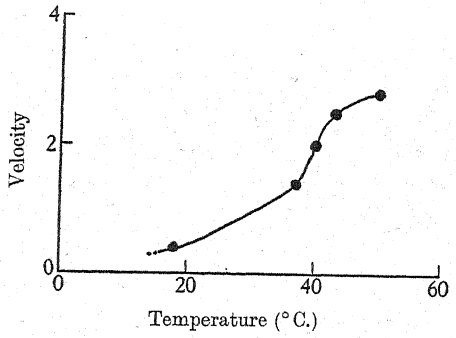

Fig. 2. Effect of temperature on rate of tetrathionate reduction. Velocity of tetrathionate reduction is expressed as the absorption of I_2 (as ml. of $0{\cdot}005N$ I_2/1 ml. sample) after incubation for 20 min. at the different temperatures.
Each tube contained washed suspension of *Bact. paratyphosum* B, $0{\cdot}01M$ tetrathionate, $0{\cdot}2M$ buffer (*p*H 7·7) and $0{\cdot}02M$ mannitol. Total vol.: 5 ml.

higher temperatures the Q_{10} falls again, and this may possibly be associated with enzyme destruction, which is rapid at temperatures above 50°. Fig. 3 shows the effect of preliminary heating of a bacterial suspension at 56°. It can be seen that the activity is reduced by over 50 % after only 10 min. treatment.

Effect of p*H*. The *p*H curve (Fig. 4) is of course a composite curve of at least two enzyme systems and corresponds to the *p*H curve of many known dehydrogenases [Cook & Alcock, 1931]. Accurate

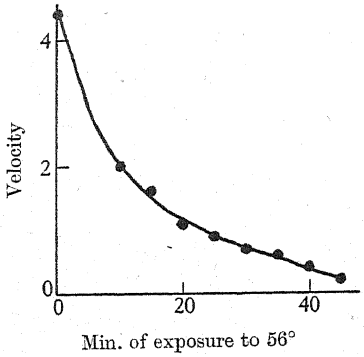

Fig. 3. Rate of inactivation of tetrathionate-reducing system at 56°. Initial velocity of tetrathionate reduction is expressed as absorption of I_2 (as ml. of 0·005 *N* I_2/2 ml. sample) after incubation for 30 min. at 37°.

Each tube contained washed suspension of *Bact. paratyphosum* B (exposed to 56° for varying periods from 0 to 45 min.), 0·01 *M* tetrathionate, 0·2 *M* buffer (*p*H 7·7) and 0·02 *M* mannitol. Total vol.: 5 ml.

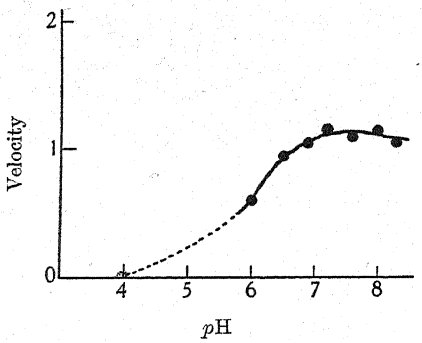

Fig. 4. Effect of *p*H on tetrathionate reduction by *Bact. paratyphosum* B. Velocity of tetrathionate reduction is expressed as absorption of I_2 (as ml. of 0·005 *N* I_2/2 ml. sample) after incubation for 30 min. at 37°.

Each tube contained bacterial suspension, 0·01 *M* tetrathionate, 0·2 phosphate buffer (*p*H 6·0–8·3) and 0·02 *M* mannitol. Total vol.: 10 ml.

results below *p*H 6·0 have not been obtainable, but when the reaction ceases in an unbuffered suspension mixture, the *p*H is about 4·0. It can therefore be assumed that the velocity below *p*H 4·0 is 0.

Effect of oxygen. Facilities were not available for testing the effect of known O_2 tensions, but Table 4 shows the relative velocities of tetrathionate reduction: (1) in evacuated Thunberg tubes, (2) in tubes open to the air, (3) in sealed tubes exposed to an atmosphere of O_2, and (4) in tubes through which

Table 4. *Effect of oxygen on tetrathionate reduction by* Bact. paratyphosum *B*

Treatment	Velocity of tetrathionate reduction (absorption of I_2, as ml. of 0·005 *N* I_2, by 2·5 ml. samples after 30 min. incubation)
(1) Anaerobic	7·2
(2) Open to air	6·85
(3) Exposed to O_2	5·75
(4) 'Oxygenated'	0·85

Each tube contained bacterial suspension, 0·01 *M* tetrathionate, 0·2 *M* buffer (*p*H 7·7) and 0·02 *M* mannitol. Total vol.: 2·5 ml. Incubation at 37°.

O_2 was bubbled continuously. The first three tubes showed little difference in velocity, probably because in all three cases the conditions were nearly anaerobic, since diffusion of O_2 from the atmosphere was not rapid enough to replace the O_2 in solution which was consumed [see Rahn & Richardson, 1941]. On the other hand, when O_2 was continuously bubbled through the mixture, tetrathionate reduction was decreased by about 90 %.

Specificity of tetrathionate reduction

The ability to reduce tetrathionate actively to thiosulphate is restricted to certain groups of organisms. For investigating the distribution of this property in different bacterial groups we used the following technique in order to provide conditions as near optimal as possible for a wide range of organisms: 18 hr. broth cultures of organisms to be tested were centrifuged and the deposit incubated with 0·01 *M* tetrathionate, 0·2 *M* phosphate buffer and 50 % tryptic digest broth as source of H_2 donators. Under these conditions the following organisms were found to reduce tetrathionate actively: *Bact. paratyphosum* B and many other *Salmonellas* (but excluding *Bact. paratyphosum* A), *Bact. typhosum* (*Eberthella typhosa*), *Proteus vulgaris* and *P. morganii*, and some intestinal non-pathogens of the coliform intermediate group. We were unable to detect any tetrathionate reduction by *Escherichia coli* I, many of the non-pathogens commonly met with in human faeces, dysentery bacilli, *Corynebacterium diphtheriae* and a few strains of streptococci and staphylococci. *Pseudomonas aeruginosa* (*Ps. pyocyanea*) occupies an interesting position, since it was found to reduce tetrathionate slowly and is reported by Starkey [1934, 1935*a*] to oxidize thiosulphate to tetrathionate.

DISCUSSION

The bacterial reduction of tetrathionate is of considerable biological interest and may have important implications in several different fields of

investigation. The relevance of tetrathionate reduction in the understanding of selective media for the diagnosis of suspected typhoid or Salmonella infections has been discussed elsewhere [Knox, Gell & Pollock, 1943]. We there suggested that tetrathionate might act as an alternative H_2 acceptor to O_2. If this is so we should expect that under anaerobic conditions tetrathionate would favour the growth of those organisms which can reduce it. In this way tetrathionate would be similar to other H_2 acceptors such as nitrate [Quastel, Stephenson & Whetham, 1925], but would be more specific and in that sense selective, because the power to reduce tetrathionate is more restricted. We have given some experimental results which suggest that this is so [Knox *et al.* 1943].

Tetrathionate reduction may be an important link in the natural cycle of synthesis and breakdown of S compounds by bacteria. Though this has not been studied so thoroughly as the N_2 cycle, there has accumulated in recent years some evidence indicating how H_2S is oxidized to sulphur and sulphate and converted to organic sulphur compounds by certain micro-organisms and then re-evolved by subsequent reduction of these substances by other bacteria [see summary by Bunker, 1936]. Beijerinck [1900, 1904] mentioned that bacteria in his group '*Aerobacter*', which contained gas-forming intestinal organisms, were able to reduce tetrathionate, pentathionate, thiosulphate, sulphite and sulphur to H_2S. It is now clear that production of H_2S from tetrathionate by bacterial action occurs, at least with the organisms that we have investigated, only when all the tetrathionate has first been reduced to thiosulphate, and that this reduction is much more rapid than the subsequent reduction of thiosulphate to H_2S. $Na_2S_2O_3$ is known to be present in many soils [Starkey, 1935*b*] and the discovery that it can be rapidly formed from tetrathionate by bacterial action is an interesting counterpart to the already well-established fact that thiosulphate can be oxidized to tetrathionate by certain heterotrophic soil organisms, amongst which are members of the genus *Pseudomonas* [Starkey, 1935*a*]. The widespread occurrence in soils and inland waters of *Ps. fluorescens* [Waksman, 1931] may have some connexion with the long survival of *Bact. typhosum* and Salmonellas outside the human body, since their supply of an alternative H_2 acceptor to O_2 (i.e. tetrathionate) would then be continuously renewed.

The reduction of tetrathionate resembles in many ways the reduction of other H_2 acceptors (e.g. nitrate) by bacteria and the mechanism of H_2 transfer is probably similar. O_2 inhibits the reduction of nitrate [Stickland, 1931]: it has also a definite though less marked inhibitory effect on the reduction of tetrathionate. In both cases there appears to be a competition between H_2 acceptors for activated H_2. That the reduction of tetrathionate need be no subsidiary means of substrate oxidation is suggested by the speed of the reaction. In one experiment the rate of H_2 transfer was equivalent to an O_2 consumption of 68 mm./mg. dry bacterial wt./hr. (assuming all the O_2 to be reduced to H_2O)—a figure which is of the same order as that for the O_2 consumption of *Esch. coli* [Cook & Haldane, 1931].

It is suggested that the system responsible for the bacterial reduction of tetrathionate to thiosulphate be named provisionally 'tetrathionase'.

SUMMARY

1. Washed suspensions of *Bacterium paratyphosum* B can reduce tetrathionate rapidly and quantitatively to thiosulphate according to the equation

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3.$$

2. This reaction can be followed simply by direct iodometric titration.

3. The power of actively reducing tetrathionate appears to be restricted to certain intestinal organisms.

4. The effect of *p*H, temperature and O_2 tension on the reaction have been investigated.

5. Some biological implications of bacterial reduction of tetrathionate have been discussed.

We should like to express our thanks to Prof. M. E. Delafield and Prof. G. S. Wilson for their valuable advice and criticism, and to Prof. C. R. Harington for his interest and encouragement.

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A Note on the Fate of Stibophen in the Body

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By means of a simple and rapid polarographic technique [Goodwin & Page, 1943] we have been able to determine the rate of excretion of Sb after the injection of stibophen (Fouadin; sodium antimony[III] *bis*-pyrocatechol-3:5-disulphonate) with a higher degree of accuracy than has previously been possible. It was considered that a critical examination of the relative rates of excretion of the Sb and the Na catechol disulphonate portions of the stibophen molecule would throw some light upon the fate of the drug in the body.

Khalil [1936 *a*, *b*] made use of the colour reaction given by catechol with iron salts to detect the presence of stibophen in the urine of patients receiving intramuscular injections of the drug. The urine was treated with a solution of $FeCl_3$ or ferrous ammonium sulphate and made strongly alkaline with NH_4OH. The depth of colour produced corresponded approximately with 'the amount of Sb in the urine as revealed by the Reinsch test'. However, the excretion of Sb continued after the colour test on the urine had become negative. Hassan [1937, 1938], using the Beam & Freak [1919] method for the determination of Sb, found that not more than 20 % of the Sb administered as stibophen was excreted in 24 hr. by schistosomiasis patients. In the present work, the rates of urinary excretion of the Sb and catechol fractions of the stibophen molecule were measured in normal human subjects by accurate methods of assay.

EXPERIMENTAL

Determination of Sb. Sb was determined in the urine by the direct polarographic method previously described [Goodwin & Page, 1943].

Determination of catechol. For the accurate estimation of the catechol portion of the molecule, Khalil's test had to be modified, as the quality and intensity of the colour was found to be affected by normal urine constituents.

When the ferrous tartrate reagent described by Mitchell [1923] was used, a clear colour unaffected by normal urine constituents was produced, and this reagent was therefore chosen in preference to $FeCl_3$ or ferrous ammonium sulphate. The catechol compound in a concentration of 1:10,000 in urine produced a measurable depth of colour, and the colour produced by Na catechol disulphonate was identical qualitatively and quantitatively with that produced by an equivalent amount of stibophen.

General procedure. Young normal male volunteers received intramuscular or intravenous injections of stibophen (6·3 % solution) or Na catechol disulphonate (4·66 % solution). Urine was collected hourly for the first 6 hr. after injection, and subsequently in 6–12, 12–24, and 24–48 hr. fractions.

To 5 ml. of urine in a test tube were added 5 ml. of a tenfold dilution of concentrated NH_3 solution (sp. gr. 0·880), and the mixture was heated for a few minutes in a boiling water-bath to coagulate any precipitate formed. After centrifuging, 2 ml. of the supernatant fluid were mixed with 2 ml. of a freshly prepared solution containing 0·1 % $FeSO_4$ and 0·5 % Rochelle salt, diluted to 10 ml., and the colour intensity estimated with a Hilger 'Spekker' absorptiometer. A blank in which the reagent was replaced

Table 1. *The urinary excretion of Sb and catechol by normal human subjects after the injection of stibophen*

Injection		% dose excreted (hr.) 0–1	1–2	2–3	3–4	4–5	5–6	6–12	12–24	24–48	Total excretion in 6 hr. %
Stibophen (5 ml. of a 6·3 % solution (41 mg. Sb) intravenously (6 subjects))	Sb:										
	%	6·0	1·9	1·6	0·5	0·6	0·6	2·3	3·3	4·4	11·2
	Range	4·1–9·9	0·9–3·7	0·5–4·1	0·3–0·8	0·2–1·0	0·2–1·0	1·3–3·6	2·0–5·5	2·8–6·2	
	Catechol:										
	%	33	23	16	10	9	7	—	—	—	98
	Range	22–55	13–47	14–22	4–14	6–12	3–9	—	—	—	
Stibophen (5 ml. of a 6·3 % solution (41 mg. Sb) intramuscularly (5 subjects))	Sb:										
	%	4·4	1·8	0·9	0·5	0·4	0·2	1·6	2·1	3·2	8·2
	Range	3·3–5·7	1·4–2·7	0·4–1·6	0·3–0·8	0·2–0·7	0·2–0·4	0·9–2·2	1·6–2·7	2·6–3·9	
	Catechol:										
	%	25	22	13	11	11	6	—	—	—	88
	Range	15–49	13–35	10–16	8–15	6–16	0–9	—	—	—	
Na catechol-3:5-disulphonate (5 ml. of a 4·66 % solution (equivalent to 6·3 % stibophen) intravenously (3 subjects))	Catechol:										
	%	29	26	16	8	8	5	—	—	—	92
	Range	20–38	18–33	8–24	5–11	6–11	2–12	—	—	—	

by H_2O was used to compensate for the colour of the original urine. The concentration of catechol was calculated by reference to a standard curve prepared with urinary solutions of stibophen of known concentrations.

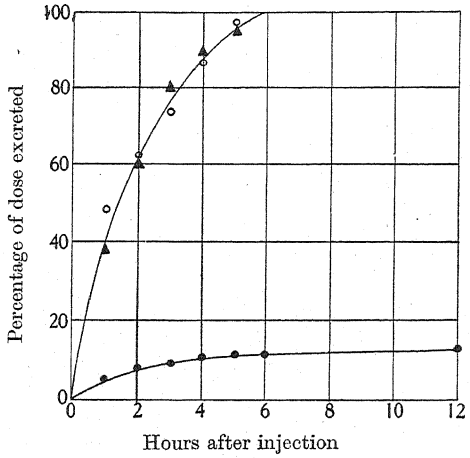

Fig. 1. The excretion of stibophen by subject M.C. • antimony, o catechol, both from a 5 ml. dose of stibophen (6·3 %). ▲ catechol, from a dose of sodium catechol disulphonate equivalent to 5 ml. of stibophen (6·3 %).

RESULTS

The results (Table 1, Fig. 1) show that in the normal subject catechol excretion was almost complete in about 6 hr., whereas the Sb excretion was very much slower, and followed a different course. The rates of excretion were not affected by changes in urine volume. In Khalil's experiments, his colour test failed after 6 hr. because the catechol portion of the molecule had been completely eliminated. The function of the catechol appears to be to keep the circulating Sb in solution in a non-toxic form while it is being absorbed by the liver or excreted by the kidney. Meanwhile, excretion of the catechol takes place independently of that of the Sb and (as shown by the results) at the same rate as that of an equivalent dose of Na catechol disulphonate.

SUMMARY

1. Khalil's test for stibophen in urine has been modified for use as a quantitative method for the determination of the catechol fraction of the molecule.

2. The catechol fraction has been shown to be excreted very much more rapidly than the Sb of stibophen, elimination being complete in six hours in the normal subject.

Our thanks are due to the volunteers, especially Mr E. Rogers, who was also responsible for the catechol determination.

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The Action of Amino-acids and Proteins on Liver-fat Deposition

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Any explanation of the lipotropic action of proteins must take into account the now well-established facts that while cystine accelerates, methionine partially prevents the deposition of fat in the livers of rats maintained on diets high in fat and low in lipotropic factors. Agreement on the question as to whether the effects of these two amino-acids alone account for the lipotropic behaviour of proteins has, however, yet to be reached. Thus Best & Ridout [1940] and Channon, Manifold & Platt [1940] conclude that these two amino-acids are not the only factors involved; in contrast, Tucker, Treadwell & Eckstein [1940] claim that the cystine and methionine contents of the diets used by them suffice adequately to explain their results. More recently, Treadwell, Groothuis & Eckstein [1942] arrived at similar conclusions but also found that when methionine was present as the free amino-acid in the diet it appeared to be superior to that contained in protein (caseinogen) so far as lipotropic action is

concerned. Before final conclusions with regard to the action of proteins on liver-fat deposition can be drawn, it is essential to have information concerning both the qualitative nature and the intensity of the action of all the naturally occurring amino-acids. We have previously reported the results of studies of a number of these acids [Beeston & Platt, 1939] and the present paper records further experiments with other pure amino-acids. At the same time the question of the lipotropic activity of proteins has been attacked from a new angle by the use of fractionated protein hydrolysates. The results of these experiments, which are also presented, indicate the complex nature of the problem.

EXPERIMENTAL

General

The animal experiments and the analyses of liver fat were carried out with rats as experimental animals, according to methods developed in this laboratory [Channon, Manifold & Platt, 1938*b*, 1940]. The basal diets used to produce fat- or cholesterol-fatty livers varied in detail in the different experiments and are mentioned as each is described. Blood fibrin was prepared according to the method of Channon, Loach, Loizides, Manifold & Soliman [1938*a*].

Estimation of amino-acids

Determinations of the cystine and methionine contents of the proteins and the amino-acid fractions were carried out by the method of Baernstein [1936]; in the titration method we have found that the recovery of methionine was 91%, which agrees with the correction figure of 1·12 given by Kassel & Brand [1938]. The tyrosine contents of the proteins and fractions prepared from hydrolysates were determined by the method of Lugg [1937, 1938].

Preparation of amino-acids

The tyrosine used in these experiments was prepared from caseinogen, the cystine from hair; other amino-acids were obtained commercially.

Preparation of protein fractions

Caseinogen hydrolysate. Caseinogen was hydrolysed with H_2SO_4 and the hydrolysate neutralized with $Ba(OH)_2$ [Berg & Rose, 1929], filtered and concentrated *in vacuo*. The product was partly solid and partly an uncrystallizable syrup.

Butanol fractionation. A caseinogen hydrolysate prepared as above was concentrated until the volume was equivalent to 1 l. for every 500 g. original caseinogen. The solution was allowed to cool, and the residue obtained by filtration is referred to subsequently as Fraction I. The filtrate was then exhaustively extracted with equal volumes of butanol on a shaking machine at room temperature. The butanol extracts were concentrated *in vacuo* and the amino-acids filtered, washed with ether and dried. The yield of butanol-soluble amino-acids was roughly 20% of the weight of caseinogen used. Butanol-insoluble amino-acids remained in the form of a syrup on concentration and were used as such.

Copper salt fractionation. The butanol-soluble amino-acids were divided into three groups, according to their solubilities in H_2O and dry CH_3OH, by the method of Town [1928, 1936]. These three groups are: (*a*) amino-acids with Cu salts insoluble in H_2O; (*b*) amino-acids with Cu salts soluble in H_2O but insoluble in dry CH_3OH; (*c*) amino-acids with Cu salts soluble in H_2O and in dry CH_3OH.

Assessment of results

Difficulties were encountered in deciding on the significance of differences which occurred in the amounts of liver fat of groups of rats receiving various dietary treatments. With carefully selected groups, each of not less than ten animals, there may be variation in the amount of fat deposited in the pooled livers even when the groups are under identical dietary treatment. These differences are greater when the livers become very fatty, and contain amounts of fat greater than, say, 1·5 g., as compared with the normal figure of 0·13 g. The differences, however, decrease rapidly as the amount of fat falls from these very high figures, so that when the liver fat is no more than three or four times the normal, they tend to disappear. Similarly with animals receiving supplements of substances under test; if the liver fat is very high, only substantial decreases can be regarded as significant. As the liver fat diminishes to 0·5–1·0 g., however, decreases of small magnitude become significant, for the ease of removal of fat from the liver diminishes as the amount of its contained fat decreases. Although methods of statistical analysis have been applied, we have found cases in which results which have subsequently been found to be significant have not been revealed so by mathematical methods. Considerable caution in interpreting apparent changes between control and experimental groups has, therefore, to be exercised, and even with great experience some degree of uncertainty in interpreting the results is inevitable.

RESULTS

Experiments with pure amino-acids

The first two experiments were designed to test the action on liver fat deposition of as many amino-acids as were available. In Exp. 1, five groups of rats were maintained on basal diet A (Table 1). Four groups received supplements of 0·2% aspartic acid, lysine, phenylalanine and serine respectively. In Exp. 2, 14 groups of rats received basal diet B (Table 1). The 12 experimental groups received supplements of 0·5% alanine, arginine, aspartic acid, histidine, hydroxyproline, leucine, lysine, phenylalanine, proline, serine, tryptophan and valine respectively.

Table 1. *Composition of basal diets (g./100 g.)*

(In addition to the following constituents all diets contained 1 g. cod-liver oil and 5 g. salt mixture/100 g. diet.)

Diet	Casein-ogen	Albu-min	Glu-cose	Beef dripping	Mar-mite	Chole-sterol	Aneurin μg./rat/day
A	5	0	46	40	3	0	0
B	0	10	44	40	0	0	10
C	5	0	64	20	5	0	0
D	0	8	54	30	0	2	10
E	0	8	46	40	0	0	10
F	10	0	45	39	0	0	12
G	0	5	50	39	0	0	12

The amino-acids tested in Exp. 1 have clearly had no action on the level of liver fat (Table 2). In Exp. 2 also, no amino-acid save tryptophan has caused the weight of liver fat to differ from the control figure by a significant amount (Table 2).

Table 2. *The effect of dietary supplements of amino-acids on liver fat of the rat*

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
	Exp. 1. Basal diet A. Period of feeding 14 days. Amino-acid supplement = 0·2%				
1	None	− 5	4·8	14·54	0·70
2	Aspartic acid	−13	4·5	15·87	0·72
3	Lysine	−13	4·7	13·60	0·64
4	Phenylalanine	− 3	4·6	14·57	0·67
5	Serine	− 7	4·5	15·06	0·67
	Exp. 2. Basal diet B. Period of feeding 18 days. Amino-acid supplement = 0·5%				
6	None	0	5·6	23·93	1·43
7	None	− 3	6·0	23·87	1·51
8	Alanine	0	5·0	23·54	1·25
9	Arginine	+ 2	4·9	24·85	1·28
10	Aspartic acid	+ 2	5·0	24·63	1·24
11	Histidine	+ 1	5·7	27·62	1·58
12	Hydroxyproline	+ 3	5·6	26·95	1·51
13	Leucine	+ 3	5·0	23·39	1·22
14	Lysine	+ 1	5·8	25·95	1·58
15	Phenylalanine	− 3	5·6	24·25	1·43
16	Proline	+ 3	5·6	22·03	1·30
17	Serine	0	4·7	27·26	1·26
18	Tryptophan	0	4·8	20·69	1·05
19	Valine	− 3	5·8	25·97	1·53

The decrease caused by tryptophan from 1·50 to 1·05 g. is noteworthy, but unfortunately further supplies of tryptophan were not available, and the necessary confirmation of this result could not, therefore, be sought.

Glutamic acid. Exps. 3–10 were concerned with a more detailed study of the effect of glutamic acid on the cholesterol- and fat-fatty liver. It was found that glutamic acid exerted some effect in preventing glyceride deposition in the cholesterol-fatty liver, but to our surprise had little significant effect on the fat-fatty liver (Table 3).

The results of Exps. 3 and 4 show that although 0·24% glutamic acid and its mono-sodium salt had little, if any, action on the levels of liver fat, 2·4% of both of these substances definitely decreased the glyceride fraction. The decreases in Exp. 3 from 1·12 g. for the control group to 0·70 g. for the 2·4% glutamic acid group, and in Exp. 4 from 0·57 to 0·38 g. in the corresponding groups, thus show that this amino-acid possesses some lipotropic action. The results of Exp. 5 agree with those of Exps. 3 and 4 and define the effect of glutamic acid more closely. Thus the effect shown by 3% of the Na salt is shown equally well at the lower level of 0·5%. A demonstrable effect is therefore shown by a 0·5% supplement of glutamic acid, but no further effect

Table 3. *Effect of glutamic acid on the cholesterol-fatty liver*

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fatty acids: In 100 g. fresh liver g.	Fatty acids: In liver of 100 g. rat g.
	Exp. 3. Basal diet C; period of feeding 17 days				
20	None	− 7	5·3	21·03	1·12
21	0·24% glutamic acid	− 6	4·6	20·08	0·91
22	0·4% glutamic acid	−10	4·5	15·58	0·70
	Exp. 4. Basal diet C; period of feeding 21 days				
23	None	− 5	4·3	13·24	0·57
24	0·24% mono-sodium glutamate	− 8	4·1	13·53	0·56
25	2·4% mono-sodium glutamate	−11	4·1	9·25	0·38
	Exp. 5. Basal diet D; period of feeding 18 days				
26	None	− 5	5·6	25·34	1·42
27	0·5% mono-sodium glutamate	− 4	5·1	21·44	1·09
28	3·0% mono-sodium glutamate	−10	4·8	20·65	0·99
	Exp. 6. Basal diet D; period of feeding 17 days				
29	None	− 8	6·7	31·88	2·15
30	0·5% glutamic acid	− 4	5·8	26·19	1·53
31	2·5% glutamic acid	− 5	5·7	27·95	1·59
32	10% glutamic acid	− 6	5·4	26·44	1·44
33	0·58% mono-sodium glutamate	− 7	5·9	31·06	1·84
34	2·88% mono-sodium glutamate	− 7	5·6	30·40	1·69
35	11·5% mono-sodium glutamate	− 6	5·7	28·91	1·65
36	1·44% $NaHCO_3$	− 3	6·4	31·47	2·00

is induced by increasing the amount of the supplement above this level.

The results of Exp. 6 show that glutamic acid has decreased the fat deposition in groups 30–32, although the fact that the decrease does not run parallel with the amount of the supplement is again noteworthy. The results for groups 33–35, in which the mono-sodium salt was used, also show some decrease in the amount of fat deposited in the 100 g. rat liver, while group 36 has served as a further control to group 35. While the effects of glutamic acid in preventing glyceride deposition in the cholesterol-fatty liver are not of great magnitude, the results, which are in agreement, are significant.

The results of Exps. 7–10 show conclusively that neither glutamic acid nor mono-sodium glutamate have any action on the fat-fatty liver. In Exp. 7 glutamic acid has raised the fat content of the liver of the 100 g. rat 0·70–0·98 g., but this result was not substantiated in Exps. 8–10, where in no case do the animals on supplemented diets show any difference from those on the control diet (Table 4).

Tyrosine. At first sight the results (Table 5) would seem to indicate that tyrosine possesses a small lipotropic action, the level of liver lipids (of the 100 g. rat) having been lowered by 0·34 g. in 1·01 g. by 0·5% tyrosine in Exp. 12 (groups 52

Table 4. *Effect of glutamic acid on the fat-fatty liver*

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
	Exp. 7. Basal diet A; period of feeding 14 days				
37	None	−5	4·8	14·54	0·70
38	0·2% glutamic acid	+1	4·6	21·56	0·98
	Exp. 8. Basal diet E; period of feeding 18 days				
39	None	−4	4·1	14·80	0·60
40	5% mono-sodium glutamate	−5	4·2	13·98	0·58
	Exp. 9. Basal diet E; period of feeding 18 days				
41	None	−2	4·9	19·34	1·01
42	3% mono-sodium glutamate	−2	4·8	20·79	1·07
	Exp. 10. Basal diet E; period of feeding 16 days				
43	None	+2	5·9	28·64	1·68
44	0·5% glutamic acid	−1	5·8	29·66	1·72
45	2·5% glutamic acid	−1	5·8	31·36	1·80
46	10% glutamic acid	+2	5·4	30·07	1·62
47	0·58% mono-sodium glutamate	+4	5·6	29·91	1·68
48	2·88% mono-sodium glutamate	0	6·1	28·58	1·75
49	11·5% mono-sodium glutamate	0	5·5	28·25	1·55

and 53). This result was substantiated in Exp. 13, where 0·3% tyrosine lowered the liver lipids (of the 100 g. rat) from 1·15 g. in group 54 to 0·71 g. in group 55. 3·0% tyrosine, however, lowered the

Table 5. *Effect of tyrosine on the fat-fatty liver*

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
	Exp. 11. Basal diet A; period of feeding 14 days				
50	None	−5	4·8	14·54	0·70
51	0·2% tyrosine	−6	4·7	12·23	0·57
	Exp. 12. Basal diet E; period of feeding 18 days				
52	None	−2	4·9	19·34	1·01
53	0·5% tyrosine	0	4·2	15·85	0·67
	Exp. 13. Basal diet F; period of feeding 18 days				
54	None	−5	4·8	24·02	1·15
55	0·3% tyrosine	−7	4·1	17·58	0·71
56	3·0% tyrosine	−2	4·4	21·10	0·93
	Exp. 14. Basal diet G; period of feeding 18 days				
57	None	−5	6·5	30·11	1·96
58	0·1% methionine	−6	6·0	31·04	1·87
59	0·3% tyrosine	−8	6·4	32·39	2·06
60	0·1% methionine + 0·3% tyrosine	−4	5·3	28·44	1·50

liver lipids by only 0·22 g. When these results were statistically analysed, the figures for the tyrosine groups were not significantly different from those for the control group. Exps. 11 and 14 do not give any indication of a possible lipotropic action of tyrosine. It would appear, therefore, that tyrosine has little if any action on the liver lipids of animals with fat-fatty livers.

Table 6. *Effect of tyrosine on the cholesterol-fatty liver*

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fatty acids: In 100 g. fresh liver g.	Fatty acids: In liver of 100 g. rat g.
	Exp. 15. Basal diet C; period of feeding 17 days				
61	None	− 7	5·3	21·03	1·12
62	0·3% tyrosine	− 2	4·3	16·41	0·71
63	3·0% tyrosine	−10	4·3	13·62	0·58
	Exp. 16. Basal diet C; period of feeding 21 days				
64	None	− 5	4·3	13·24	0·57
65	0·3% tyrosine	− 6	3·9	10·98	0·43
66	3·0% tyrosine	− 7	3·9	11·21	0·43
	Exp. 17. Basal diet D; period of feeding 18 days				
67	None	− 5	5·6	25·34	1·42
68	0·5% tyrosine	− 8	5·6	26·31	1·47
	Exp. 18. Basal diet D; period of feeding 16 days				
69	None	− 6	7·1	33·11	2·35
70	0·5% tyrosine	− 4	7·2	30·33	2·18
71	None	− 6	8·2	34·73	2·83
72	0·5% tyrosine	0	8·2	33·83	2·76

In only one of these four experiments with the cholesterol-fatty liver does tyrosine exert any significant effect on the liver lipids (Table 6), this being in Exp. 15, where 0·3% tyrosine lowers the liver lipids of the 100 g. rat from 1·12 g. in group 61, to 0·71 g. in group 62, and 3·0% tyrosine lowers the level to 0·58 g. (group 63). In the other three experiments tyrosine exerts no significant effect on the amount of liver fat. These findings appear to confirm the above conclusion that tyrosine lacks any lipotropic activity. In one experiment (Exp. 14) 0·3% tyrosine and 0·1% methionine together have exerted an action on the level of liver lipids which is greater than that exerted by 0·3% tyrosine or 0·1% methionine alone, and it was this result which led to a belief that in conjunction with other amino-acids tyrosine may influence the level of liver lipids. In later experiments, therefore, when amino-acid fractions of equivalent methionine and cystine content were compared for action on liver lipids, the tyrosine contents were also equalized, so that the true effect of amino-acids other than these three should become apparent.

Experiments with protein hydrolysates

It was now becoming obvious that the problem of the lipotropic action of proteins should be tackled from another angle, and the most obvious method was the examination of the action of various fractions of the protein molecule, formed by hydrolysis on the liver lipids of animals with fatty livers, since it is plain from the work of Channon *et al.* [1940] that cystine and methionine alone cannot explain the lipotropic action of proteins.

Casein hydrolysate. Up to the present there has been only one recorded observation concerning the lipotropic action of a protein hydrolysate. Carter & Melville [1940] found that hydrolysed caseinogen, with tryptophan added to replace that lost during hydrolysis, had the same activity as the original caseinogen. The amount of the amino-acid added appears to have been arbitrary, and no actual determinations were made. In this work, however, no account was taken of the possible loss of cystine and methionine during hydrolysis. We have found that as much as 50 % of these amino-acids may be lost during hydrolysis by the method employed. This result is, therefore, of doubtful value.

We, therefore, compared the lipotropic action of unhydrolysed caseinogen with that of hydrolysed caseinogen to which methionine and cystine and tyrosine were added in amounts to ensure an intake of these amino-acids equal to that provided by the original caseinogen. Possible effects of tryptophan were, for the present, ignored.

Three groups of rats (73–75) received basal diet G. Group 73 served as control, group 74 received a supplement of 15 % caseinogen, and group 75 the hydrolysis products equivalent to a 15 % supplement of caseinogen with the addition of sufficient cystine, methionine and tyrosine to bring the intakes of these amino-acids up to the level of the caseinogen group. The analytical figures concerning the caseinogen and its hydrolysate are recorded in Table 7.

Table 7. *Amino-acid content of caseinogen and hydrolysed caseinogen*

Material	Total content (g.) of Solids	Nitrogen	Cystine	Methionine	Tyrosine
Caseinogen	315	40·05	1·55*	8·37*	18·60*
Total hydrolysis products of 315 g. caseinogen	269	36·50	0·60	6·86	12·26
Recovered after hydrolysis (% of total)	85	81	39	82	66

*. Based on the following percentage contents: cystine 0·5; methionine 2·75; tyrosine 6·0.

Table 8. *Effect of hydrolysed caseinogen on the fat-fatty liver*

Exp. 19. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
73	None	−9	5·95	33·94	2·02
74	15 % caseinogen	+7	3·98	13·49	0·54
75	Hydrolysate of 15 % caseinogen	−5	3·85	14·28	0·56

The results (Table 8) show that supplemented hydrolysed caseinogen has the same lipotropic action as the unhydrolysed caseinogen, for even though the animals receiving the hydrolysate lost 5 % of their weight, while those receiving caseinogen gained 7 %, previous experience shows that these differences are not of sufficient magnitude to influence the liver-fat deposition. Thus the conclusion to be drawn from this experiment is that if there is present in caseinogen one or more compounds which affect liver-fat deposition, other than cystine, methionine and tyrosine, these further compounds are stable to acid hydrolysis. Since tryptophan is in part destroyed during hydrolysis, this result implies that this amino-acid plays only a minor, if any, part in liver-fat deposition.

Butanol fractions of caseinogen hydrolysates. At this point we tested the action of fractionated caseinogen hydrolysates, and the groups of amino-acids soluble or insoluble in butanol were chosen as the most readily prepared fractions for the initial experiments.

When the action of caseinogen and the butanol-soluble and insoluble fractions of the amino-acids derived from it were compared, additions of cystine, methionine and tyrosine were again made so that the intake of these 'active' amino-acids was the same as that in the group receiving caseinogen. It also was considered desirable to equate the N intake with that of the caseinogen group; suitable amounts of gelatin were therefore added, since gelatin has been shown to be without lipotropic action except at high levels [Best, Grant & Ridout, 1936; Channon *et al.* 1938*a*]. An additional group received 10 % gelatin also supplemented to bring the intake of the 'active' amino-acids to the same level as that of the caseinogen group. A further group received blood fibrin, since it was desirable to obtain more information regarding the action of this protein than had previously been obtained [Channon *et al.* 1938*a*].

The analytical data for the various amino-acids, and for the protein fractions used in this experiment, are presented in Table 9. The basal diet G (Table 1) was used and was supplemented in the five experimental groups with 10 % caseinogen (77), 6 % butanol-soluble amino-acids and 4 % gelatin (78), the butanol-insoluble amino-acids equivalent to 10 % caseinogen with gelatin sufficient to equate the N intake to that of group 77 and 78 (79), 10 % gelatin (80) and 10 % blood fibrin in group 81. Cystine, methionine and tyrosine were added to groups 77–81 in amounts sufficient to ensure that the intake of these amino-acids in these groups was equivalent.

Table 10 shows that the addition of 10 % caseinogen has lowered the liver fat from the control figure of 1·96 g. (group 76) to 0·75 g. in group 77, while

Table 9. *Amino-acid content of proteins and of amino-acid mixtures used in Exp. 20*

Material	Content (%) of Methionine	Cystine	Tyrosine
Gelatin	0·5	0·1	0·0
Blood fibrin	2·0	1·5	4·4
Caseinogen	2·75	0·5	6·0
Butanol-soluble amino-acids (yield: 20% of original caseinogen)	4·6	0·5	4·1
Butanol-insoluble amino-acids from 500 g. caseinogen (233 ml. of a non-crystallizable syrup containing 189 g. solids)	0·61	0·29	0·91

Table 10. *Effect of various proteins and supplemented fractions of hydrolysed caseinogen on the fat-fatty liver*

Exp. 20. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
76	None	−8	6·51	30·16	1·96
77	10% caseinogen	+6	4·28	17·58	0·75
78	6% butanol-soluble amino-acids	−3	3·50	10·42	0·37
79	3·8% butanol-insoluble amino-acids	+3	4·72	24·44	1·15
80	10% gelatin	−1	4·05	19·72	0·80
81	10% blood fibrin	+8	4·87	21·35	1·06

in group 78, which received the butanol-soluble amino-acids, the amount of liver fat is even lower at 0·37 g.; while the difference between these two latter figures is not striking, it is statistically significant. Since groups 77 and 78 both received the same intake of the amino-acids known to be active, it is thus a reasonable deduction that the butanol-soluble fraction contains either more of a further amino-acid which decreases liver fat or less of one which accelerates liver-fat deposition than that present in caseinogen. This conclusion seems supported by the results for group 79 (Table 10), which received the butanol-insoluble amino-acid fraction, for while the amount of liver fat (1·15 g.) is again not strikingly different from that for the caseinogen group 77 (0·75 g.), the difference is in the reverse direction from that of the butanol-soluble group and is of similar magnitude. The fact that the amount of butanol-soluble amino-acids fed was three times greater than their yield from 10% caseinogen does not, of course, affect this conclusion, although it is impossible to deduce more from these findings than that the lipotropic action of caseinogen may not be accounted for by the actions of its contained cystine, methionine and tyrosine.

In apparent contrast with this conclusion are the results for group 80, which received gelatin which was also supplemented with cystine, methionine and tyrosine. Gelatin supplemented in this way, so that the intakes of the above amino-acids were the same as in group 77, had an action indistinguishable from caseinogen, even though it is known to be without action when unsupplemented. While the results of this experiment seem in the light of previous experience to be convincing, their admittedly unexpected nature made it desirable to confirm them, and Exp. 21 was, therefore, carried out under the exact conditions of Exp. 20, save that the action of fibrin was not investigated further.

Table 11. *Effect of proteins and supplemented fractions of hydrolysed caseinogen on the fat-fatty liver*

Exp. 21. Basal diet G; period of feeding 17 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
82	None	−10	5·40	26·78	1·45
83	10% caseinogen	+ 1	4·16	14·05	0·59
84	6% butanol-soluble amino-acids	− 6	3·66	9·63	0·35
85	3·8% butanol-insoluble amino-acids	− 1	4·38	18·88	0·83
86	10% gelatin	− 2	3·91	17·01	0·67

The results of this experiment (Table 11) confirm in every particular those of Exp. 20. Particularly interesting from the immediate point of view is the confirmation of the finding that when gelatin, which alone is devoid of lipotropic action, is supplemented with cystine, methionine and tyrosine, its action is indistinguishable from that of caseinogen. It will be remembered that in groups 78 and 79 of Exp. 20, as well as in groups 84 and 85 of the present experiment, it was considered desirable to equate the N intake to that of the caseinogen groups, and for this purpose gelatin, a protein known to be lipotropically inactive, was used. As a result, the immediate conclusion from the previous and present experiments that it is possible to prepare from caseinogen hydrolysates fractions which, irrespective of their contents of cystine, methionine and tyrosine, exert actions of varying magnitude on liver-fat deposition, may not be valid because of the possible interfering action of the gelatin supplements. We therefore compared with that of caseinogen the action of a similar amount of gelatin alone, and supplemented with cystine, methionine and tyrosine in amounts which were such that the intake of these amino-acids was similar to those of the caseinogen group; at the same time study was made of the action of similar amounts of these amino-acids alone.

Basal diet G (Table 1) was supplemented as follows: group 95, 15% caseinogen; group 98, 15% gelatin; group 99, 15% gelatin plus sufficient cystine, methionine and tyrosine to make the level

of these amino-acids up to that present in the caseinogen supplement of group 95; group 100, cystine, methionine and tyrosine in amounts equivalent to those in the 15 % caseinogen supplement.

Table 12. *Effect of gelatin and some caseinogen fractions on the fat-fatty livers*

Exp. 23. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
94	None	−9	5·95	33·94	2·02
95	15 % caseinogen	+7	3·98	13·49	0·54
96	2·6 % fraction I	−3	3·74	13·44	0·50
97	9·8 % fraction II	−4	4·15	14·38	0·59
98	15 % gelatin	−7	5·38	33·09	1·78
99	15 % gelatin + cystine, methionine and tyrosine	−4	3·53	13·15	0·46
100	Cystine, methionine and tyrosine equivalent to 15 % caseinogen	−4	3·65	14·74	0·54

A comparison of the liver-fat deposition in group 95 (Table 12) with that in group 99 provides yet further confirmation that the action of gelatin, when supplemented with active amino-acids, is indistinguishable from that of an equivalent weight of caseinogen. The results for group 98 demonstrate again that unsupplemented gelatin is without effect. The result of most immediate interest is that given by group 100, where it is very clearly demonstrated that an intake of an amount of cystine, methionine and tyrosine equal to that present in a 15 % caseinogen supplement has an action identical with that of a 15 % caseinogen supplement itself. This result suggests that the lipotropic action of caseinogen depends solely on its content of these three amino-acids, and that it was purposeless to pursue the investigation further. On the other hand, the findings in Exps. 20 and 21 seem to be in conflict with this view. In both experiments the amounts of fat deposited in the livers of the groups receiving the butanol-soluble and insoluble fractions differ not only from each other, but also from that deposited in the caseinogen groups, by amounts which are statistically significant. This conclusion is supported by the findings of Exp. 23, which show that the additions of gelatin made in groups 78 and 79 of Exp. 20 and in groups 84 and 85 of Exp. 21, which were made in order to equate the N intakes to that of the caseinogen groups, has had no influence on the liver-fat deposition. If this be so, something other than cystine, methionine and tyrosine must account for the differences resulting from the administration of the fractions soluble and insoluble in butanol, and the investigation was therefore carried further.

Cu salt fractions of casein hydrolysate. In Exps. 20 and 21 the butanol-soluble amino-acids were fed as a 6 % supplement, an amount chosen because it equated, without further supplement, the methionine intake of the groups receiving it with that of the caseinogen groups. In order to attempt to obtain a more quantitative comparison with caseinogen this fraction was now fed in the amount which would be derived from the weight of caseinogen with which it was being compared; this procedure had been adopted in the case of the butanol-insoluble amino-acids in both Exps. 20 and 21 and was again repeated for this fraction. No attempt was made to equate the N intake for the reasons already discussed, and the use of gelatin was, therefore, discontinued.

At the same time it was felt desirable to compare further fractions from the butanol-soluble amino-acids with the unfractionated acids, since the results of Exps. 20 and 21 suggested that if there did exist one or more unknown lipotropically active constituents, the study of this fraction was likely to be most profitable. For this purpose the Cu salt separation described in the experimental section was used and three fractions of amino-acids prepared: (*a*) with Cu salts insoluble in H_2O, (*b*) with Cu salts soluble in H_2O and insoluble in dry CH_3OH, and (*c*) with Cu salts soluble in H_2O and soluble in dry CH_3OH.

Methionine, cystine and tyrosine estimations were carried out on each fraction and the analytical data for these three fractions and for the butanol-soluble and insoluble amino-acids are presented in Table 13.

Table 13. *Amino-acid contents of the fractions of caseinogen used in Exp. 22*

Material	Content (%) of Cystine	Methionine	Tyrosine
(*a*) Butanol fractionation			
Caseinogen	0·5	2·75	6·0
Butanol-soluble amino-acids (20 % of caseinogen)	0·3	5·1	4·0
Butanol-insoluble amino-acids (183 g. from 500 g. caseinogen)	0·46	0·88	1·15
(*b*) Cu salt fractionation of butanol-soluble amino-acids (125 g.)			
Amino-acids with Cu salts insoluble in H_2O (42 g.)	0·43	5·34	4·40
Amino-acids with Cu salts soluble in H_2O and insoluble in CH_3OH (28 g.)	0·28	5·68	4·17
Amino-acids with Cu salts soluble in H_2O and soluble in CH_3OH (32 g.)	0·11	1·28	4·05
Total recovered after fractionation* (g./100 g. starting material)	76·0	66·5	86·0

* The total weight of the three fractions was 102 g., i.e. 82 % of the weight of the starting material.

Although neither Town [1928, 1936] nor Caldwell & Rose [1934] claim that the Cu salt method gives a clean separation of amino-acids into three groups, the figures given in Table 13 for the cystine,

methionine and tyrosine contents of the three fractions show that in our hands the separation is not good. Town [1941] has recently stated that the presence of NH_3 and of the dicarboxylic amino-acids greatly interferes with the Cu salt separation, and that these substances must be removed before attempting the fractionation. Since this was not done in these investigations, it may account for the poor separation of the Cu salts of the above-mentioned amino-acids.

Basal diet G (Table 1) was supplemented as follows: group 88, 15% caseinogen; group 89, 3% butanol-soluble amino-acids, which represented the weight of these amino-acids derived from 15% caseinogen; group 90, 5·6% butanol-insoluble amino-acids, equivalent to 15% caseinogen; groups 91–93, the amino-acids obtained from the different Cu salt fractions of the butanol-soluble fraction in amounts derived from 3% of the latter, and thus equivalent to 15% caseinogen. In addition, groups 89–93 received supplements of methionine, cystine and tyrosine in amounts calculated on the basis of the analyses recorded in Table 13, so that these groups obtained the same intake of these acids as did group 88, which received 15% caseinogen.

Table 14. *Effect of caseinogen and fractions thereof and of fractions of the butanol-soluble amino-acids on the fat-fatty liver*

Exp. 22. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
87	None	−9	4·79	26·52	1·27
88	15% caseinogen	+5	3·96	10·12	0·40
89	3% butanol-soluble amino-acids	−4	3·54	8·61	0·31
90	5·6% butanol-insoluble amino-acids	0	3·60	14·00	0·51
91	1% amino-acids with Cu salts insoluble in H_2O	−8	4·02	11·56	0·47
92	0·7% amino-acids with Cu salts soluble in H_2O and insoluble in CH_3OH	−6	4·39	21·61	1·00
93	0·8% amino-acids with Cu salts soluble in H_2O and soluble in CH_3OH	−6	3·93	16·75	0·66

The results (Table 14) confirm the presence in caseinogen of a factor or factors other than cystine, methionine and tyrosine, which plays some part in the lipotropic action of caseinogen. In spite of the fact that groups 88–93, inclusive, received equivalent intakes of cystine, methionine and tyrosine, the amounts of fat deposited in the livers of rats varied from 21·6% in group 92 to 8·6% in group 89.

These results introduce two possibilities: that there exists in caseinogen either a substance with a methionine-like action which is concentrated in the butanol-soluble amino-acids and from there into the group of amino-acids having Cu salts insoluble in H_2O, since this group (91) has an action indistinguishable from caseinogen, or a substance with a cystine-like action, which cannot exert this action until it is separated—as for example by the copper-salt method—from some inhibiting factor. The result of group 92 lends some belief to the latter idea since the level of liver fat in this group is very much greater than that in the caseinogen group (89), although both groups received equivalent intake of the known active amino-acids.

From an examination of the analytical data for the butanol-soluble and insoluble groups of amino-acids, it will be seen that together these make up only a little over 57% of the original caseinogen, thus leaving approximately 43% unaccounted for. The action of this fraction on liver fat was investigated in Exp. 23. Two fractions of hydrolysed caseinogen were examined, fraction II, which is the sum of the butanol-soluble and insoluble amino-acids, and fraction I, which is the rest of the hydrolysed caseinogen. The hydrolysate from 315 g. caseinogen was concentrated *in vacuo* to 650 ml. and allowed to crystallize. The amino-acids which separated were filtered off and dried. This comprised fraction I. The filtrate was concentrated as far as possible by vacuum distillation, when about two-thirds of the material was obtained in the solid form and the rest in the form of a non-crystallizable syrup. The solids plus syrup constituted fraction II. Cystine, methionine and tyrosine determinations were carried out on these fractions and the analytical data recorded in Table 15.

Table 15. *Amino-acid contents of the fractions of hydrolysed caseinogen used in Exp. 23*

Material	Amounts: g.	Amounts: % of starting material	Total content (g.) of: Cystine	Methionine	Tyrosine
Caseinogen	315	100	1·55	8·37	18·60
Fraction I	52	17	0·10	3·41	8·84
Fraction II	206	65	0·39	3·11	2·09
Fractions I and II	258	82	0·49	6·55	10·39
Total recovered after hydrolysis (% of that originally present)	—	82	32	78	59

Groups 96 and 97 were supplemented by fractions I and II respectively in amounts equivalent to a 15% supplement of caseinogen. Groups 96 and 97 were supplemented with cystine, methionine and tyrosine in amounts such that the intakes of these amino-acids were equivalent to that of a 15% caseinogen supplement (group 95). The results for these groups have already been recorded in Table 12.

Clearly the amounts of fat in the livers of animals in groups 95, 96 and 97 are indistinguishable, and the results support those for groups 95, 98, 99 and

100, in showing that cystine, methionine and tyrosine alone may account for the lipotropic action of caseinogen.

We have now arrived at the point where two series of experiments have indicated opposing solutions to the problem of accounting for the lipotropic activity of caseinogen.

DISCUSSION

The results of Exps. 20–22 make it clear that cystine, methionine and tyrosine alone cannot explain the lipotropic activity of caseinogen, since groups of rats receiving equivalent intakes of the above amino-acids have liver-fat levels which are significantly different. Furthermore, these differences are large enough for the conclusion to be drawn that there exists in the protein molecule some new factor which affects the level of liver lipids, and that this factor varies in amount in the different fractions examined. Although in Exps. 20 and 21 the results are complicated by the presence of gelatin, in Exp. 22 this is not the case, and here the differences are clearly demonstrated. The butanol-soluble amino-acids have a greater lipotropic action than an equivalent amount of caseinogen. Furthermore, the figures for the liver-fat levels of the groups of animals fed the amino-acids fractionated by the Cu salt method show very distinct differences, even though the cystine, methionine and tyrosine intakes were equivalent in the three groups.

The experiments with pure amino-acids have given no clue to a further factor which affects liver fat. Of the 14 amino-acids examined so far, none has shown any significant effect. Interesting in itself, although casting no light on the general problem, was the observation that glutamic acid exerts a lipotropic action on the cholesterol-fatty liver but not on the fat-fatty liver. There would appear to be no adequate explanation of this fact at present, but it may be bound up with the fact that both caseinogen and egg albumin, which were used as basal proteins, contain considerable amounts of glutamic acid, and at the levels at which they are fed contribute over 1 % of that amino-acid to the diet. Glutamic acid is, therefore, the only substance which has so far been encountered in this laboratory which exerts an effect on the cholesterol-fatty liver and yet is without action on the fat-fatty liver. Tryptophan was found in one experiment to possess only a small lipotropic action, and this is not sufficient evidence for any concrete conclusion to be drawn about this amino-acid. Tyrosine also has a questionable action on liver fat.

Singal & Eckstein [1941] found that *dl*-valine, *dl*-leucine and *dl*-isoleucine had no effect on liver-fat deposition. In addition, it has been found in this laboratory that glycine also has no action on the fatty liver [Manifold, 1939], so that the only amino-acids which have not been examined are threonine, nor-leucine, nor-valine and hydroxyglutamic acid, and the possibility exists that one of these may show significant action on the fatty liver.

The conclusions to be drawn from the results obtained with supplemented gelatin in Exps. 20, 21 and 23 would appear to be in opposition to that already drawn from Exps. 20–22, and indicate that cystine, methionine and tyrosine alone account for the lipotropic activity of caseinogen. Tucker *et al.* [1940] came to a similar conclusion when they found that the lipotropic action of a low-protein (5 % caseinogen or edestin), high-fat diet, so supplemented with cystine and methionine that its content of these amino-acids was equal to that of a 20 % caseinogen diet, was similar to that observed when the level of caseinogen in the diet was 20 %. On the other hand, Best & Ridout [1940] found that cystine and methionine in amounts equivalent to a 30 % caseinogen supplement had little lipotropic action compared with that of a 30 % caseinogen supplement. Now Channon *et al.* [1940] showed that for both free cystine and methionine there is a limiting intake above which no further increase in the amounts of these amino-acids ingested had any influence on the level of liver fat, and these authors came to the conclusion that the action of cystine and methionine alone cannot explain the lipotropic activity of caseinogen.

A finding which further complicates this work is that of Treadwell *et al.* [1942], whose experimental data seem to indicate that diets supplemented with methionine, as the free amino-acid, were superior in lipotropic action to the diets containing equivalent amounts of methionine in the form of protein. Such a finding casts a certain amount of doubt on the conclusion drawn from the result of Exp. 23 (and of parts of Exps. 20 and 21) concerning the supplemented gelatin.

The balance of this work appears to be in favour of the existence of at least one unknown factor in caseinogen which affects the level of liver fat. The results with the butanol-soluble amino-acids would lead one to assume the presence of a lipotropic factor, whereas the results of Exp. 22 (groups 91, 92 and 93) lead to the conclusion that there exists another substance, with a cystine-like action, which is not manifest in the presence of certain other protein constituents.

It is abundantly clear that much further work is necessary before any definite conclusions may be drawn concerning this problem. A further factor which may influence the action of fractions of hydrolysed caseinogen is that we have no knowledge of the fate of the amino-acids which are partially destroyed during hydrolytic and other treat-

ments. The possibility exists that they may be converted into substances which have actions on the fatty liver different from that of the amino-acids in the original form. An example of this reversal of action is given by the work of Singal & Eckstein [1941], who found that whereas cystine accelerated liver-fat deposition, cystine betaine had the opposite action, and another substance having a lipotropic action was dithiodiglycollic acid.

It is to be regretted that conditions did not allow of our continuing this investigation, and consequently our results are presented in an incomplete form.

SUMMARY

1. Fourteen pure amino-acids were investigated with a view to ascertaining whether or not they had any effect on the deposition of fat in the livers of rats on diets high in fat and low in lipotropic factors.

2. Tryptophan was found to possess a small lipotropic action in one experiment, and tyrosine to possess a small but questionable action on the fat- and cholesterol-fatty livers.

3. Glutamic acid was found to exert a lipotropic action on cholesterol- but not on fat-fatty livers.

4. The lipotropic actions of various protein fractions were studied. Evidence was obtained that some factor which affects liver-fat deposition, other than cystine, methionine and tyrosine, is present in the protein molecule. This factor can be concentrated in the butanol-soluble amino-acid fraction. Fractionation by the copper-salt method effected further concentration of the unknown factor and indicated the possible presence of a substance which accelerates liver-fat deposition. Conflicting results have been obtained in experiments with gelatin, and in one experiment with caseinogen fractions, and more work is necessary to elucidate the problem completely.

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A Biological Method for Estimation of Pantothenic Acid with Rats, in which Wheat Germ is included in the Basal Diet

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Investigations on the B_2 group of vitamins made with chicks fed on a heated diet of mixed grains, and with rats on synthetic diets supplemented by purified aneurin (vitamin B_1), riboflavin, and pyridoxine (vitamin B_6), have led to the discovery that the filtrates obtained when extracts of yeast, liver or rice bran are treated with Fuller's earth contain substances essential for growth of these animals [Lepkovsky, Jukes & Krause, 1936; Elvehjem & Koehn, 1935]. These substances, generally known as filtrate factors, were called factor 2 by Lepkovsky and his colleagues. Chicks deprived of filtrate factors are checked in growth and develop a characteristic dermatitis; young rats also show subnormal increase in weight and a less specific skin affection with greying of the pigmented fur. In due course

it was discovered that the filtrate factor fraction contained a substance identical with 'pantothenic acid', a compound found necessary for the growth of certain strains of yeast [Williams, Lyman, Goodyear, Truesdail & Holaday, 1933]. For a concise account of the gradual discovery to date of the different components of the vitamin B_2 complex reference should be made to the review by Lepkovsky [1942].

Before the identification and synthesis of pantothenic acid was accomplished [Williams & Major, 1940], Jukes [1937] had measured the 'filtrate factor' potency of different foods by feeding them to chicks maintained on the heated grain diet. The criterion adopted was rate of weight increase, a preliminary attempt to use degree of cure of dermatitis having proved unsatisfactory.

There is no record of any attempts to use rats for the biological estimation of pantothenic acid, although the growth-promoting activity of certain materials fed to rats as supplements to the so-called 'filtrate-deficient' diet has been measured in this laboratory [Copping, 1943]. The results cannot be applied directly to the pantothenic acid content of these materials, since it is now certain that 'filtrate factor deficiency' is not a deficiency of pantothenic acid alone, but also of at least one other growth-promoting substance [see Lepkovsky, 1942].

In an attempt to estimate a vitamin by biological method, in which the growth of a young animal is adopted as criterion, the first requisite is a basal diet which shall contain, in optimum amounts, all other essential nutrients. We therefore looked for an addition to the 'filtrate-deficient' synthetic diet which should supply the additional unknown growth 'filtrate factors' without pantothenic acid. Our first choice was a whole liver extract in which we hoped to destroy the pantothenic acid present by acid or alkaline hydrolysis. This proved unsuccessful (see below, p. 494), and we finally chose wheat germ. Bakke, Aschehoug & Zbinden [1930] noted the development of greyness of rats with pigmented fur on a diet in which wheat germ supplied the B vitamins, and ascribed the effect to toxicity of the wheat germ. Miss A. M. Copping (private communication) made a similar observation while measuring the growth response of young pied rats to doses of wheat germ added to a 'filtrate-factor deficient diet', the rats becoming grey while still growing. It was assumed that this was due to the absence of pantothenic acid in wheat germ, a material which nevertheless contained considerable amounts of the other additional growth factor or factors. Preliminary tests based on this assumption have met with some success, and in this paper is given an account of these and of the development of a biological method suitable for estimation of pantothenic acid, in which the growth of young rats is the criterion adopted.

EXPERIMENTAL

The rats, all males of the usual Lister pied strain, were weaned on to a basal diet deficient in all B_2 vitamins for 1–2 weeks before receiving the test doses. The basal diet was that employed at the Lister Institute, and, except for a few preliminary tests in which starch was given, the carbohydrate was present in the form of sucrose. The composition was as follows: sucrose 60, casein 20, arachis oil 12, salts 5 and lard 3 parts. Vitamin B_1 as pure aneurin hydrochloride was given daily, 15 μg. to each rat, and cod-liver oil to supply vitamins A and D. The diet was unsupplemented with any B_2 vitamins for a period of 2 weeks in the hope that any variations due to reserve of stores of these vitamins in the young rat's body might be lessened by this procedure.

The rats, which usually weighed 40–55 g. at the beginning of the tests, were subsequently weighed three times a week. At the beginning of the test period they were put into separate cages of the usual pattern, distributed evenly by weight and litter among the various groups. They then received supplements of B_2 vitamins other than pantothenic acid in pure form. Two solutions of mixed vitamins were prepared. The first contained 30 μg. aneurin, 30 μg. pure vitamin B_6 (pyridoxine) and 50 μg. riboflavin in the daily dose of 2 ml. The second was stored in a dropping bottle which delivered, in the daily dose of 2 drops, 1 mg. inositol, 1 mg. nicotinic acid and 3 mg. choline chloride. Although it is doubtful whether the rat requires inositol and nicotinic acid, their inclusion in the supplements seemed desirable.

The basal sugar diet was put into heavy glass dishes and pressed down firmly to avoid scattering. If scattering took place, water was added, and any diet remaining unconsumed after 24 hr. was discarded. Fresh dry diet was given daily in weighed amount to supply a slight excess over the rat's requirements.

When wheat germ was given, the daily dose was weighed into a heavy glass dish, moistened with the drops of inositol, nicotinic acid and choline chloride; this mixture was usually consumed readily before any basal diet was given. With the larger doses of wheat germ, extra water was needed. The doses of vitamin B_1, vitamin B_6 and riboflavin were given in the same dish. The doses of pure pantothenic acid, given to the control series of rats, were from a solution containing 100 μg./ml. of calcium-*d*-pantothenate.

The rats were kept on the test doses for 4 weeks, weighed three times a week, and any symptoms noted as they appeared. The absence of symptoms was also recorded when the test was finished.

RESULTS

Experiments with whole-liver extract

Since pantothenic acid is reported to be unstable to acid and alkaline hydrolysis it was expected that a source of the unidentified factors free from it might be obtained by subjecting liver extract to these treatments.

Accordingly a whole-liver extract was adjusted to *p*H 0 by the addition of conc. H_2SO_4 and boiled on a sand bath for 10 hr. The resulting dark solution was neutralized with baryta, filtered under pressure,

Table 1. *Growth of rats receiving supplements of liver extract with or without added pantothenate*

Exp.	Supplementary dose, daily	No. of rats	Average weekly weight increase (g.) Week 1	Week 2	Week 3	For whole period
1	Whole liver extract ≡ 6 g. fresh liver	6	27·0	25·7	24·0	25·6
	„ acid treated	8	20·0	17·8	18·9	18·9
	„ acid treated + 75 μg. pantothenate	7	22·0	21·6	21·2	21·6
2	Whole liver extract ≡ 6 g. fresh liver	3	33·0	24·0	27·9	28·3
	„ alkali treated	4	30·7	22·5	21·2	24·8
	„ alkali treated + 75 μg. pantothenate	4	33·0	28·8	28·2	30·0

extracted twice with ether in an attempt to remove bitter-tasting products of hydrolysis and freed from ether by evacuation. The growth-promoting effect of this extract with and without pantothenic acid was compared with that of the untreated extract, but it was clear that the pantothenic acid had been only partially destroyed by the treatment, since addition of pantothenate to the treated extract gave but a small improvement in weight increase which was not statistically significant (Table 1, Exp. 1).

Alkaline hydrolysis was carried out in solution raised to *p*H 11·5 by addition of baryta. The first attempts produced a dark-coloured product with bitter taste, which was badly taken by the rats and caused diarrhœa. Finally, a palatable and innocuous product was obtained after hydrolysis for 5 hr. at this *p*H. The growth on the dose given was, however, sufficiently near to that on the original liver extract to indicate that little destruction of pantothenic acid had occurred, and the increased growth produced by the addition of pantothenic acid was too small to form the basis of an assay, although statistically significant (see Exp. 2, Table 1).

Experiments with wheat germ

To find out whether wheat germ was deficient in pantothenic acid but contained the additional growth factors, 23 rats from six litters were divided into three groups and given daily doses, respectively, of 1 g. wheat germ, 1 g. wheat germ plus 150 μg. calcium-*d*-pantothenate, and 150 μg. calcium-*d*-pantothenate only.

The results summarized in Table 2 show quite clearly that when 1 g. wheat germ daily was fed with 150 μg. calcium-*d*-pantothenate, an amount known [cf. Unna, 1940] to be in excess of the rat's requirements, the average weekly weight increase (27·1 g.) was greater than with pantothenic acid

Table 2. *Growth of rats receiving supplements of wheat germ with or without added pantothenate*

Exp.	Supplementary dose, daily	No. of rats	Average weekly weight increase (g.) Week 1	Week 2	Week 3	Week 4	For whole period
3	1 g. wheat germ	7	22·6	19·4	15·6	11·6	17·3
	1 g. wheat germ + 150 μg. Ca-*d*-pantothenate	8	24·4	26·0	27·6	30·4	27·1
	150 μg. Ca-*d*-pantothenate	8	22·1	17·9	17·3	—	19·1
4	0·5 g. wheat germ	7	18·4	14·4	9·8	11·8	13·6
	0·5 g. wheat germ + 100 μg. pantothenate	8	27·5	27·4	29·2	37·2	30·3
	1 g. wheat germ	8	22·1	13·3	12·9	14·5	15·7
	1 g. wheat germ + 100 μg. pantothenate	8	27·4	24·0	26·6	25·2	28·3
	2 g. wheat germ	8	23·8	18·6	20·0	24·0	21·6
	2 g. wheat germ + 100 μg. pantothenate	8	26·7	25·9	23·9	26·3	25·7
	Whole liver extract ≡ 6 g. fresh liver	5	31·6	23·8	30·1	—	28·5

Table 3. *Growth of rats receiving supplements of pantothenate, liver extract and/or wheat germ*

Exp.	Supplementary dose, daily	No. of rats	Average weekly weight increase (g.) Week 1	Week 2	Week 3	Week 4	For whole period
5 (*a*)	Liver extract ≡ 12 g. fresh liver	4	34·0	30·8	39·6	34·4	34·7
	Liver extract ≡ 6 g. fresh liver + 1 g. wheat germ	4	32·5	34·1	36·0	41·0	35·9
	Liver extract ≡ 6 g. fresh liver + 2 g. wheat germ	3	32·7	30·3	36·0	38·6	34·4
5 (*b*)	Liver extract ≡ 6 g. liver	8	29·1	28·1	29·5	26·5	28·3
	Liver extract ≡ 3 g. liver + 0·25 g. wheat germ	8	30·9	25·1	28·3	32·1	29·1
	Liver extract ≡ 3 g. liver + 0·5 g. wheat germ	8	28·4	27·8	28·4	29·4	28·5

All rats received 100 μg. Ca-*d*-pantothenate daily.

alone (19·1 g.), thus affording evidence for the presence in wheat germ of the additional unknown growth-promoting substances. Without pantothenic acid the growth-promoting effect was considerably reduced (average weekly weight increase 17·3 g.), demonstrating the deficiency of wheat germ in this substance (Exp. 3, Table 2).

In order to determine how much wheat germ was needed to meet the rat's daily requirements, doses of 0·5, 1 and 2 g. daily were chosen and tested with and without 100 μg. pantothenic acid. Fifty-two rats from ten litters were used in this test, and a group of rats receiving whole-liver extract served as a positive control (Exp. 4, Table 2). These results showed that 0·5 g. wheat germ was as effective as 1 or 2 g. in growth-promoting power when supplemented with 100 μg. pantothenic acid, the average weekly weight increases being 30·3, 28·3 and 25·7 g. respectively. Increasing amounts of wheat germ fed without pantothenic acid gave increasing growth rates. This might be explained by the assumptions: (*a*) that 0·5 g. wheat germ contains more than the rat's daily requirement of the additional factors, and/or (*b*) that wheat germ also contains a small amount of pantothenic acid. The maximum weekly weight increase in this series, 30·3 g. when 0·5 g. wheat germ was supplemented with 100 μg. pantothenic acid, is comparable with that obtained, 28·5 g., when whole-liver extract equivalent to 6 g. of fresh liver was given.

In order to determine whether wheat germ supplies all the additional growth factors other than pantothenic acid, comparison was made between wheat germ and whole-liver extract, which is the richest known source of these factors and was used by Macrae, Todd, Lythgoe, Work, Hind & El Sadr [1939]. For this test 34 male rats from 11 litters were used. If wheat germ and whole-liver extract each contain all these additional factors and were given in adequate amounts, either should give maximum growth, and any mixture of the two should be equivalent to a corresponding amount of either. This was tested when 2 g. wheat germ were compared with whole-liver extract equivalent to 6 g. fresh liver, and also when 0·5 g. wheat germ was compared with liver extract equivalent to 3 g. fresh liver, calcium-*d*-pantothenate and other vitamins being fed at optimum doses to all animals. The last tests were important, because, if differences existed between wheat germ and liver extract with regard to the relative proportions of the hypothetical additional factors, such differences would be more apparent at lower dosage levels. No evidence, however, was found for any qualitative differences or, within the limits of the test, for differences in the proportions of the additional factors. The results are given in Table 3.

Effect of additions of different amounts of pantothenic acid to 0·5 g. wheat germ. A daily dose of 0·5 g. of wheat germ was chosen as suitable, and determinations were made of the effect on growth rate of additions of 0, 12·5, 25, 50, 100 and 200 μg. daily of calcium-*d*-pantothenate. These results are divided into three sections, since litters containing six male rats were not always obtainable for all the levels to be tested at once.

The pantothenate was made up in two solutions containing, in 1 ml., 25 and 50 μg. calcium-*d*-pantothenate, respectively, and a little chloroform was added. The requisite amounts were delivered into the dose dish before it was placed in the cage.

The results in Table 4 show that 12·5–50 μg. pantothenate daily were suboptimal doses, and that the optimal dose was probably between 50 and

Table 4. *Growth of rats receiving supplements of wheat germ with varying amounts of added pantothenate*

Exp.	Supplementary dose, daily	No. of rats*	Average weekly weight increase (g.) Week 1	Week 2	Week 3	Week 4	For whole period
6 (*a*)	0·5 g. wheat germ	18	16·1	11·5	9·3	9·5	11·6
and	0·5 g. wheat germ + 12·5 μg. Ca-*d*-pantothenate	18	19·8	14·4	12·9	14·1	15·3
6 (*c*)	0·5 g. wheat germ + 25 μg. Ca-*d*-pantothenate	19	20·2	20·2	18·7	10·9	17·5
	0·5 g. wheat germ + 100 μg. Ca-*d*-pantothenate	18	27·8	26·6	27·2	29·6	27·8
	Liver extract ≡ 6 g. fresh liver	6	26·5	17·9	18·7	24·5	26·9
6 (*b*)	0·5 g. wheat germ	14	16·2	10·4	9·1	9·1	11·2
and	0·5 g. wheat germ + 25 μg. Ca-*d*-pantothenate	13	24·0	18·1	15·9	17·9	19·1
6 (*c*)	0·5 g. wheat germ + 50 μg. Ca-*d*-pantothenate	14	23·9	23·3	23·6	23·2	23·5
	0·5 g. wheat germ + 100 μg. Ca-*d*-pantothenate	14	29·2	24·2	27·3	29·3	27·5
6 (*c*)	0·5 g. wheat germ	8	15·6	11·8	9·5	8·3	11·3
	0·5 g. wheat germ + 12·5 μg. Ca-*d*-pantothenate	8	20·0	16·8	13·6	13·2	15·9
	0·5 g. wheat germ + 25 μg. Ca-*d*-pantothenate	8	22·9	19·7	16·2	15·2	18·5
	0·5 g. wheat germ + 50 μg. Ca-*d*-pantothenate	10	22·7	26·3	23·7	23·3	23·0
	0·5 g. wheat germ + 100 μg. Ca-*d*-pantothenate	8	31·5	25·5	29·1	28·3	28·6
	0·5 g. wheat germ + 200 μg. Ca-*d*-pantothenate	4	26·3	25·5	22·3	27·1	25·3

* The results given in Exp. 6 (*c*) for doses of 12·5, 25, 50 and 100 μg. Ca-*d*-pantothenate are included in the averages given for these doses in Exps. 6 (*a*) and 6 (*b*).

100 μg. This figure is in accord with the figure of 80 μg. daily, given by Unna [1940] as the optimal daily dose for rats. The same result emerges from Fig. 1, in which a fairly smooth curve was obtained when the average weekly weight increases calculated from the results of Exp. 6 (*c*), etc. in Table 4 were plotted against doses of pantothenate. If the

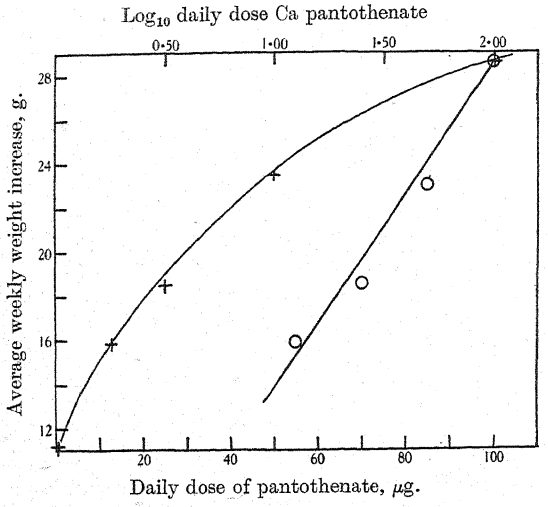

Fig. 1. Curve showing relation of average weekly rate of weight increase in young rats to the daily dose of Ca-*d*-pantothenate added to a basal diet containing other B vitamins, 0·5 g. wheat germ being given daily to each rat.

logarithms of the doses are plotted, the curve approximates to a straight line up to doses of about 100 μg. Where possible, rats from the same litter were used in the comparison of these doses, and in one series controls were included receiving a 6 g. equivalent of whole-liver extract (Exp. 6 (*a*)). Since litters containing six or more male rats occurred infrequently, comparison of the effects of the addition of 0, 12·5, 25 and 100 μg. in Exp. 6 (*a*), and of 0, 25, 50 and 100 μg. pantothenate in Exp. 6 (*b*) were made with litters containing four male rats only.

Occurrence of symptoms of deficiency of pantothenic acid

The symptoms noted when insufficient doses of pantothenic acid were given were greying of the naturally pigmented portions of the fur and also a generalized soiling and staining of the fur with an orange yellow substance, mainly in the hind ventral regions. The former is considered characteristic of pantothenic acid deficiency, the latter, which is due to a porphyrin body, has been noted in deficiency of riboflavin as well as in that of the filtrate factor of the vitamin B_2 complex [Chick, Macrae & Worden, 1940]. The above symptoms, which appeared to be aggravated when a small dose of pantothenate (12·5 μg. daily) was given, are possibly due to a greater strain caused by the increased growth stimulated; they tended to disappear when the rats received 100 μg. daily of Ca-*d*-pantothenate.

In addition, there was a tendency for the normal jet black patches of fur of all rats to turn a rusty colour after they had received wheat germ, a change not observed when whole-liver extract was given.

DISCUSSION

The use of rats for the estimation of pantothenic acid in natural materials has advantages over the microbiological method, since for the latter the complete extraction of the pantothenic acid is needed in a form in which it can be utilized by bacteria. The presence of 'bound' forms of pantothenic acid has been suggested by the work of Pennington, Snell & Williams [1940], of Waisman, Henderson, McIntire & Elvehjem [1942] and of Strong, Feeney & Earle [1941]. If it may be assumed that the rat can absorb pantothenic acid from all those forms of combination from which it is available to other animals, estimation by the method here described is particularly suitable for avoiding this difficulty.

The successful development of the method on the assumption that wheat germ supplies ample amounts of the 'additional factors' is to be taken as further proof of their existence, and the magnitude of the increase in growth rate ascribable to them suggests that they play a major part in the nutrition of the rat.

SUMMARY

1. Wheat germ has been found to contain adequate amounts of the one or more unidentified factors required by the rat, and is comparatively deficient in pantothenic acid.

2. These facts have been made the basis of a method for the estimation of pantothenic acid by the growth of young rats.

3. Addition of pantothenic acid to a basal synthetic diet supplemented by vitamin B_1 (aneurin), vitamin B_6 (pyridoxine), riboflavin, inositol, choline, nicotinic acid, cod-liver oil and wheat germ was followed by an increase in the growth rate proportional to the logarithm of the amount fed.

4. The optimum daily dose of calcium-*d*-pantothenate for a young rat (40–150 g. weight) appears to lie between 50 and 100 μg.

In conclusion we acknowledge the personal grant to one of us (J. S. D. B.) from the Medical Research Council, and gifts to the Division of Nutrition of calcium-*d*-pantothenate from Messrs G. Merck, Rahway, N.J., of pure vitamin B_1 and riboflavin from Roche Products Ltd. and of liver extract from Glaxo Laboratories Ltd. We also wish to thank Dr H. Chick for helpful advice and Miss Ruby Leader for assistance in the care of the experimental animals.

Appendix: Statistical Analysis

By J. O. IRWIN and G. N. JENKINS

By analysis of variance, the differences from litter to litter of the animals in Exp. 6 (*c*) were tested and found not to be significant. Hence it is justifiable to estimate the standard errors of mean responses or of slope from the variance in growth of animals (not littermates) on the same dose.

	b	σb	Ratio
Average increase in response (i.e. body-weight increase/week) when dose is doubled	4·26	1·12	3·80
Average increase in response when dose is increased tenfold	14·16	3·70	3·80

The standard error is based on 28 degrees of freedom and the slopes are therefore highly significant. The curve relating the logarithm of the dose to the response shown by rate of weight increase shows no significant departure from linearity.

Ignoring the addition to the error of any estimation due to the error of the estimate of slope, which may, of course, be considerable, the limits of error for one estimation with ten rats on the standard and ten on the test substance would be:

$p=0·99$	64 to 157%
$p=0·95$	55 to 181%

This basis is comparable with that used in the British Pharmacopoeia Commission Report on the Accuracy of Biological Assays [1936]* and the accuracy found with that usually obtained in biological assays.

For Exps. 6 (*a*) and 6 (*b*) the results are as follows:

Exp.	Slope b	σb	Ratio	Limits of error ($p=0·99$) for 10 rats on each dose
6 (*a*)	4·30	0·76	5·7	61 to 164%
6 (*b*)	4·19	1·02	4·1	64 to 157%

The errors in the result are increased if the period of dosing is reduced from 3 to 4 weeks, although the results are still significant.

* *Gen. Med. Coun. Brit. Pharm. Com. Report*, p. 10 [1936].

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Intermediate Stages in the Metabolic Conversion of Benzpyrene to 8-Hydroxy-benzpyrene in Mice

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Chalmers & Crowfoot [1941] showed that after administration of benzpyrene to rats and mice a small amount of monohydroxybenzpyrene could be isolated from the faeces. Berenblum, Crowfoot, Holiday & Schoental [1943] identified this as 8-hydroxy-3:4-benzpyrene, and established the presence of 3:4-benzpyrene-5:8-quinone in the faeces. Intermediate metabolites, 'BPX' and 'BPF' could be demonstrated by a blue fluorescence of the bile [Peacock, 1936, 1940] and a blue-green fluorescence of the faeces [Chalmers, 1938] respectively, both distinguishable from the violet fluorescence of the parent hydrocarbon in organic solvents. Doniach, Mottram & Weigert [1943 *a*, *b*] found that the blue 'BPX' fluorescence changed to the blue-green 'BPF' fluorescence after the contents of the small

intestine had passed through the ileo-caecal valve. Furthermore, evidence was given that a blue fluorescence with a spectrum similar to that of 'BPX' appeared in the kidney cortex, liver and lung of mice after intravenous inoculation of, or feeding with, benzpyrene, and in the skin after painting with it. The benzpyrene compounds to which the specific fluorescence was due (called collectively 'tissue-BP-blue') were produced locally in the specific organs and in the painted area of the skin. A blue-green fluorescence with a spectrum similar to that of 'BPF' was sometimes seen in the lungs of rabbits.

The typical fluorescence spectrum of 'bile-BPX' and 'tissue-BP-blue' with maxima near 450 and 425 mμ, and that of 'BPF' with maxima near 465 and 440 mμ, were seen on examination of the original tissues and of their unpurified extracts in ultra-violet light. It has now been possible, after a simplified chromatographic purification of the fluorescent compounds in these extracts, to study in greater detail the course of benzpyrene metabolism.

EXPERIMENTAL

Mice which had been inoculated in the tail vein with 1 ml. of a coarse suspension of 0·05% benzpyrene in saline were killed after 3–7 hr., and the gall-bladder and digestive tract removed. The small intestine, which fluoresced blue, and the large intestine (caecum + colon + faeces), which fluoresced blue-green, were rinsed superficially with distilled H_2O, minced, and each shaken mechanically with about 3 ml. of acetone. The bile from the opened gall-bladder was shaken in a small tube with 1 ml. of acetone. These operations were carried out as quickly as possible after death. The solids were centrifuged down and the acetone extracts transferred to benzene by adding to the clear acetone solutions about 3 ml. of benzene, and washing two or three times in a tubular separating funnel with $N/100$ H_2SO_4. The benzene solutions were then separated and dried over anhydrous Na_2SO_4.

The dried benzene solutions were poured on to columns of alumina 7–10 mm. in diam. and 3–4 cm. long, and the chromatograms developed under gravity with benzene for about 1 hr. The progress of adsorption and the behaviour of the fluorescent zones on development could easily be followed in the ultra-violet light of a G.E.C. Osira lamp with a Wood glass bulb. The fluorescent zones and rings on the Al_2O_3 were spectrographed separately with the illuminating device described by Doniach *et al.* [1943*a*], which was particularly well suited for making 'optical sections' at any place on the column. The various portions were then cut and eluted with absolute ethanol.

RESULTS

Bile and small intestine. A bluish violet-fluorescent adsorbate with a banded fluorescence spectrum was strongly fixed at the top of the column, and did not move down on development. Its ethanol eluate fluoresced blue, with essentially the same spectrum as the adsorbate. From the spectrograms graphs were recorded, using Goldberg's [1910] densograph, which served as a primitive microphotometer [Weigert & Mottram, 1940]. The graphs (Fig. 1) for the adsorbates (*a*) and the ethanol eluates (*b*) are almost identical except for a slight displacement towards longer wave-lengths for the

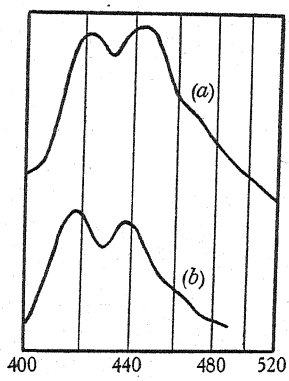


Fig. 1.

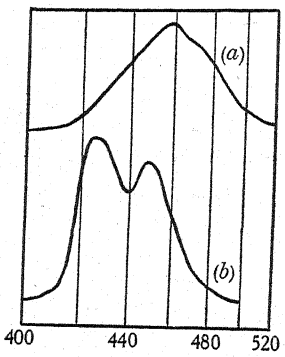


Fig. 2.

Figs. 1, 2. Photometric records of fluorescence spectrograms of mouse tissue extracts after chromatography. Abscissae: wave-length in mμ (prismatic spectrum). Ordinates: density (arbitrary scale).

Fig. 1. Bile. (*a*) Blue-fluorescent adsorbate. (*b*) Blue-fluorescent eluate in ethanol.

Fig. 2. Large intestine. (*a*) Green-fluorescent adsorbate. (*b*) Blue-fluorescent eluate in ethanol.

adsorbate. The photometric record for the adsorbate (*a*) shows two maxima, at 422 and 443 mμ, and an inflexion at about 472 mμ, which appears as a weak secondary band in the spectrum itself. The corresponding wave-lengths for the ethanol eluate (*b*) are 417, 438 and 470 mμ respectively.

Large intestine. A bright green-fluorescent ring moved slowly down; its spectrum showed one broad maximum at about 460 mμ, but no bands (Fig. 2*a*). The ethanol eluate fluoresced whitish blue with a banded spectrum. The photometric record (Fig. 2*b*)

shows two maxima, at 425 and 449 mμ, but no inflexion. Sometimes a faint blue-fluorescent adsorbate remained fixed at the surface.

Influence of storage of material before extraction. When the extraction was not carried out immediately after killing the mouse, a green-fluorescent chromatographic zone also appeared with extracts from bile and small intestine. Therefore, in another set of experiments, the diluted bile and the minced small and large intestines were each divided into two portions immediately after death, one of which was stored in ice for 3 days, while the other was extracted at once. The benzene extracts of the latter were kept over anhydrous Na_2SO_4 for 3 days until the others were ready, when they were all chromatographed together side by side in pairs. With the material stored before extraction, the blue fluorescence at the surface was greatly reduced in the case of the bile and small intestine, while a new green zone appeared and moved slowly down on development. This zone showed, both as adsorbate and as eluate, the spectrographic properties described above for the large intestine. In the case of the large intestine the phenomena were unaltered by postponement of the extraction. The change from the blue- to the green-fluorescing adsorbate with bile and small intestine was greatly accelerated when the specimens were stored at room temperature, or at 37°, before extraction.

Whenever a particularly bright green-fluorescent zone appeared, a red-fluorescing diffuse zone could be seen under it on the column; it moved down more quickly than the green zone, and could be eluted with chloroform. Neither the red adsorbate nor its eluate in chloroform showed a banded fluorescence spectrum.

The effect of preservation of the intestines by formaldehyde was also investigated. The digestive tracts were kept for periods up to 2 months in formol-saline (containing 4% H.CHO) at room temperature. Acetone extracts of the minced small intestines which had been transferred to benzene gave only the blue fluorescence at the top of the chromatographic column, while those of the large intestine gave the green-fluorescent zone which moved down slowly on development.

Properties of the different fluorescent substances. The solubility of the blue-fluorescent adsorbate in ethanol seemed to be distinctly lower than that of the green-fluorescent adsorbate. This was in agreement with the observed fact that the green zone moved slowly down on development with benzene, while the blue-fluorescent substance stayed at the top. In $N/100$ NaOH the blue-fluorescent adsorbate on the Al_2O_3 dissolved to give a solution with a banded blue fluorescence spectrum, while the alkali solution of the green adsorbate fluoresced green with a single broad maximum.

When the separated blue-fluorescent Al_2O_3 zone was dried and heated in an evacuated tube to 100–200°, the fluorescence changed from blue through white to yellow-green, and the fluorescence spectrum (which in the case of the original adsorbate was banded) changed to a spectrum with one broad maximum. An ethanol extract after this transformation fluoresced whitish blue and had the same banded spectrum as the eluate of the green zone. When the ethanol extract was transferred to benzene and again poured on to a column of Al_2O_3, a single green-fluorescent ring appeared and moved slowly down on development.

The ethanol eluate of the blue-fluorescent adsorbate from the small intestine of a single mouse contained a quantity of material sufficient to record some absorption bands. With the eluate in a quartz tube, 4 cm. long and 4 mm. diam., narrow bands appeared at 366–372, 377–382, 386–393 and 405–412 mμ. The small intestine of another mouse was kept for 1 day at 37° before extraction with acetone, when the blue-fluorescent derivative was completely transformed into the derivative which gave a green-fluorescent adsorbate on alumina. Its eluate in ethanol in a tube 1 cm. long and 4 mm. diam. showed quite different and wider bands at 358–364, 375–384, 392–407 and 415–433 mμ, with maxima at 362, 381, 400 and 422 mμ respectively.

The minced kidneys, livers and lungs of benzpyrene-treated mice were extracted with acetone immediately after killing, and the extracts transferred to benzene and chromatographed. The excess of dissolved unchanged benzpyrene, which was particularly great in the lung, was first washed out with benzene, after which development was continued for about 1 hr. Bright blue-fluorescent adsorbates remained on the column in the case of liver and kidney, and traces in the case of lung. The fluorescence spectra of these adsorbates and of their eluates were exactly the same as in the case of bile and small intestine. However, in contrast to them, the blue-fluorescent zones were not sharp but extended diffusely (about 1 cm. with kidney and about 2 cm. with liver) into the column. The lungs of three mice yielded a green-fluorescent adsorbate, which behaved like that from the large intestine.

Postponement of the extraction of kidney and liver had an effect on the chromatographic phenomena similar to that observed with bile and with small intestine. For instance, when the organs had been left for 1 day at 37° in saline, a narrow green ring moved slowly through the diffuse blue zone down the column. The rate of this movement was, in the case of liver, about twice that for kidney, but the fluorescence spectra of these green rings as adsorbates and as eluates in ethanol were the same as with the large intestine. The green zones did not appear if the organs were stored in formol-saline.

DISCUSSION

Two different kinds of fluorescence phenomena can be distinguished by chromatography in extracts of various tissues and of the bile of mice after their treatment with benzpyrene. The prototypes are, on the one hand the bile, where Peacock [1936] discovered the blue-fluorescent 'BPX', and on the other hand the large intestine and faeces, where Chalmers [1938] established the presence of the blue-green-fluorescent 'BPF'. Since it is obvious that the fluorescent zones on the Al_2O_3 contain the bulk of the fluorescent compounds in bile and faeces, it is suggested that the term [BPX] should be used for the type of the compound from bile which gives after chromatography a *banded blue* fluorescence spectrum as adsorbate and *the same* banded spectrum as eluate (Fig. 1 *a*, *b*) and the term [BPF] should be used for the type of the compound from the large intestine and faeces which gives after chromatography a *non-banded green* fluorescence spectrum as adsorbate and a *banded whitish blue* spectrum as eluate (Fig. 2 *a*, *b*).

The approximate fluorescence maxima of the four compounds before chromatography (inverted commas) and after chromatography (square brackets) may be summarized as follows:

Before	After
'BPX': 425, 450 mμ	[BPX]: 417, 438, 470 mμ
'BPF': 440, 465 mμ	[BPF]: 425, 449 mμ

[BPX] is more strongly adsorbed on Al_2O_3 and less soluble than [BPF]. They are chemically to be distinguished from each other, because their fluorescence spectra, their absorption spectra and their chromatographic behaviour are different.

The nature of [BPX] and of [BPF] has not yet been ascertained because our adsorbates contained only minute amounts. However, the fluorescence and absorption spectra of [BPX] and [BPF] and their chromatographic behaviour lead to some tentative conclusions:

(*a*) [BPF] is closely related to 8-hydroxybenzpyrene prepared by Berenblum & Schoental [1943] from faecal extracts which had been concentrated by chromatography followed by sublimation *in vacuo*. These authors were in fact working with [BPF].

(*b*) Slight differences in the rate at which the green zone moved down the chromatographic column were seen when [BPF] was prepared from different sources. Hence, [BPF] probably comprises a group of complexes which can contain various cell constituents in combination with 8-hydroxybenzpyrene.

(*c*) This combination must be a loose one, because the absorption maxima of the eluate of [BPF] in ethanol (362, 381, 400 and 422 mμ) correspond closely with the absorption maxima (362, 380, 401 and 424 mμ) read from the graph of the absorption spectrum of pure 8-hydroxybenzpyrene in ethanol published by Berenblum, Crowfoot, Holiday & Schoental [1943].

(*d*) The marked difference between the fluorescence spectra of the adsorbate of [BPF] on Al_2O_3 and of its eluate in ethanol shows that its hydroxybenzpyrene component is directly affected by the adsorption.

(*e*) [BPX] too must be considered as a group of complexes, made up of benzpyrene or a derivative and various cell constituents as carrier molecules. This follows from the different chromatographic behaviour of [BPX] from various sources.

(*f*) The fact that the same banded fluorescence spectrum is shown by all the members of the [BPX] family, as adsorbates and as eluates, indicates that the benzpyrene 'nucleus' is the same in all cases and is not affected by the adsorption. Hence the strong adsorption to the top of the column is due to the adsorbing properties of the carrier molecules.*

(*g*) The absorption bands of the ethanol eluate from [BPX] at 366–372, 377–382, 386–393 and 405–412 mμ coincide neither with those of 8-hydroxybenzpyrene nor with those of any other known benzpyrene derivative. Therefore, nothing definite can be said at present about the chemical nature of the benzpyrene derivative in the complex. However, the observed easy transformation of 'BPX' into 'BPF' in the living mouse after passage through the ileo-caecal valve or post mortem by autolysis, and of [BPX] into [BPF] *in vitro* by heat, shows that 'BPX' and [BPX] are chemical precursors of 'BPF' and [BPF] respectively.

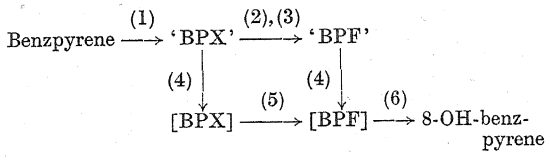
The limits of the stability of [BPX] have not yet been established: it is stable in tissues in the form of 'BPX' over long periods of time in the presence of formalin, and visible and near ultra-violet light do not alter [BPX] to a perceptible degree, either as adsorbate or as eluate.

The experiments here described, in conjunction with those of Doniach, Mottram & Weigert [1943 *a*, *b*], provide evidence for a tentative chart (shown on p. 501) of the various stages in the metabolic and chemical change from benzpyrene to 8-hydroxybenzpyrene.

The red-fluorescent derivative which appears on the alumina column when [BPF] is produced via (2) or (3) and (4) has chromatographic properties similar to those of the benzpyrene-5:8-quinone discovered by Berenblum & Schoental [1943] in the faeces, i.e. it moves down more quickly than [BPF]

* We owe the suggestion of the possible existence of a complex of a benzpyrene derivative with another strongly adsorbing substance to a personal communication from Dr Berenblum.

and can be eluted with chloroform. However, its red fluorescence is not in agreement with the properties of benzpyrene-5:8-quinone, according to Berenblum & Schoental.



(1) *In vivo*, local production in the skin, kidney, lung and liver (excreted with the bile).
(2) *In vivo* in the large intestine and occasionally in the lung of rabbits and mice.
(3) *In vitro* by post-mortem autolysis.
(4) *In vitro* by adsorption on to alumina.
(5) *In vitro* at elevated temperature *in vacuo*.
(6) *In vitro* by sublimation *in vacuo*.

Of the various steps in the transformation of benzpyrene into 8-hydroxybenzpyrene outlined above, step (1) is the only one concerned in the problem of benzpyrene carcinogenesis, because it alone occurs in the living cell. The later steps which eventually lead to hydroxybenzpyrene are merely concerned with the removal of the hydrocarbon from the body.

SUMMARY

It has been shown by fluorescence chromatography of quite fresh extracts from organs of mice which had been treated with benzpyrene that the hydrocarbon is not metabolized directly to 8-hydroxybenzpyrene but that the transformation passes through three intermediate stages which have been studied separately. The suggested relationship of these stages has been discussed.

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Effect of Vitamin B on the Growth of Fibroblasts

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Components of the vitamin B complex have long been considered of importance in growth. In particular vitamin B_1 has been assayed by animal growth methods. The experiments described were designed to test whether such a growth-promoting effect could be demonstrated directly on cells *in vitro*. In essence the problem was approached in two ways: first, by depriving fibroblasts of components of the vitamin B complex by growing them in media obtained from pigeons suffering from acute beriberi; secondly, by adding to such deficient media appropriate amounts of vitamin B (yeast extract) and vitamin B_1 (aneurin).

METHODS

Culture material. Tissue cultures of fibroblasts were obtained from the choroidal and sclerotic layers of the eyes of 9-day chick embryos, and culture by the hanging drop technique was carried out for at least ten passages prior to each experiment. This length of cultivation has been found to produce a uniform outgrowth of fibroblasts, and any initial supply of growth-promoting substance present in the tissue has been largely exhausted [Carrel & Ebeling, 1921]. Cultures which showed excellent outgrowth were then graded for an experiment. The areas of the explants were measured, and the cultures divided into sets in such a way that the explants in any two or more sets to be compared were approximately equal in area. The selection

of sets to be used for each treatment was made at random. The preparations were then mounted for the actual experiment by the double coverslip method of Maximow. Care was taken that the volume of plasma and extract used was constant and equal, that the cavity of the hollow ground slide was of the same size for all cultures, and that the size of the spread drop was the same in all cases. This last was effected by placing under each coverslip a standard circle drawn on paper, and spreading the drop to that size for each preparation.

Culture medium. The medium consisted of equal parts of the tissue extract to be tested and plasma obtained from pigeons suffering from acute beriberi ('beriberi plasma'). This plasma was used in all the experiments both for the experimental and for the control groups of cultures. In any one experiment the plasma for both experimental and control cultures was obtained from the same pigeon. Since the plasma was constant and the technique the same for all cultures of any experiment, the only varying factors which might influence growth were the tissue extracts or the vitamin preparations which were added.

Brain extracts ('beriberi brain extract') for the experimental cultures were obtained from pigeons with the classical symptoms of acute beriberi as described by Peters [1936]. These symptoms included opisthotonus in all cases except one which showed emprosthotonus (Exp. 5, pigeon used for refeeding cultures at 48 hr.). Defective vision not amounting to blindness was present; this symptom was not ascertained in the case of two birds which had typical convulsions. Weight loss averaged about 30% of the original weight of the bird. In the pigeons used the time taken to produce these effects was 24–29 days. The methods used to produce beriberi were those recommended by Kinnersley, Peters & Reader [1928]. The brain was removed as soon as possible after decapitation, and extracts were made immediately. Extracts for the control cultures were similarly prepared from the brains of normal pigeons (normal brain extract). In experiments requiring extracts of normal embryos, these were prepared by mincing fowl chicks of 8–9 days' incubation.

It was found by W. Dale and one of us that the water content of adult avian brain tissue was less than that of avian embryo, the latter containing 2·03 times the water of the former. To standardize our extracts, therefore, 2·03 cc. of Ringer-Compton-Pannett solution were added to each gram of freshly prepared brain pulp. After standing for 30 min., the diluted brain pulp or the undiluted embryo pulp was centrifuged at 1500 r.p.m. for 10 min. The supernatant fluid was regarded as 100% extract and further dilutions were made on this basis. Extracts of normal brain have been used successfully in this laboratory for some years, and have been found to provide a rich source of growth-promoting substances.

In Exp. 1, for convenience, dried extracts of brain were used throughout. An apparatus similar to that described by Flosdorf & Mudd [1935] was employed. Such dried extracts can be diluted to any required strength and are satisfactory and convenient.

Vitamin sources. The vitamin B_1 used throughout was synthetic (aneurin chloride hydrochloride) and was supplied by Prof. A. R. Todd. The concentration used was such that 0·04 mg. was present in 100 ml. of the medium in which the cultures were grown. This calculation was based on the curative dose of aneurin for a pigeon weighing 300 g. If this amount is regarded as being 3 μg./day, or 6 μg. in 2 days, then the amount in the culture medium (changed every 48 hr.) was five times the curative concentration for a pigeon.

For vitamin B an extract of brewer's yeast was used. This had been prepared for experimental purposes by Glaxo Laboratories Ltd. It was at first thought to include all the members of the B complex of vitamins found in brewer's yeast and to contain 133 μg./g. of vitamin B_1 (as aneurin chloride hydrochloride) and 154 μg./g. of riboflavin (as hydrochloride). It was later learned that most of the biotin of this preparation had remained in the residue, and that the vitamin B_1 content was unstable, and probably negligible at the time of the experiment. The amount of the extract used was based on the stated content of aneurin, and was calculated as given above for vitamin B_1. Neither the aneurin nor the yeast extract was sterilized. Both were weighed with aseptic precautions and were dissolved in the sterile distilled water used in making the Ringer solution with which the tissue extract was diluted.

The measurement of outgrowth. Experimental and control groups of cultures were incubated at 39°. After 48 hr. the outline of each explant and its outgrowth was drawn by projection. The cultures were then washed with the Ringer solution and fed with fresh extracts in the same dilution as was used in the first instance. After incubation for a further 48 hr., the new outlines were drawn. The areas obtained were measured with a planimeter, and the relative increase in size was calculated after the method described by Ebeling [1921]. The difference in the mean relative increase in two groups of cultures differently treated was examined for significance using the '*t*'-test and extensions of this test.

RESULTS

The influence of beriberi brain extracts on the growth of fibroblasts in vitro

(1) *The influence of the concentration of the extract.* The first experiment was designed to find whether,

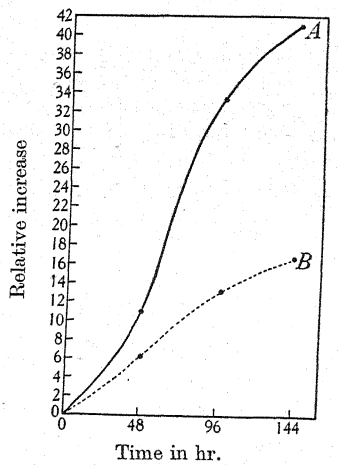

Fig. 1. Comparison of growth when two concentrations of beriberi brain extract were used. Concentration of extract in *A* was ten times that of *B*.

when vitamin B was deficient, growth-promoting factors were still present in tissue extracts. It was

argued that if growth-promoting factors were present in beriberi brain extracts, their influence would depend on their concentration, as occurs with embryo extract; if growth-promoting factors were absent then no difference in growth would occur when two concentrations were compared. To one group was added 200 %, to the other 20 % extract produced from dried extract. Measurements were made every 48 hr. Re-feeding at those times was carried out, using freshly dried material in the same dilutions as were used initially. A total of 39 cultures was used.

The group fed with 200 % beriberi brain extract grew much better than that fed with 20 %. The average relative increases are shown quantitatively in Fig. 1. The difference in the relative increase was found to be highly significant. ($t = 3{\cdot}57$ at 48 hr.; $t = 4{\cdot}41$ at 96 hr.; degrees of freedom (d.f.) = 37.) This experiment showed that extracts from brains of pigeons suffering from beriberi were still growth-promoting to fibroblasts, and that the stimulation of growth increased with the concentration of such extracts.

(2) *A comparison of the growth obtained with normal and beriberi brain extracts.* Having found that extracts from pigeons suffering from acute beriberi retained growth-promoting properties, we compared the activity of such extracts with that of extracts of normal tissue. The experimental cultures were fed with 10 % fresh beriberi brain extract; for the controls 10 % fresh normal brain extract was used. In Exp. 2 there was a total of 35 cultures, and in Exp. 3 a total of 56 cultures. In both experiments the growth of the cultures fed with beriberi brain extract was less than that in the groups fed with normal brain extract, values of $t = 1{\cdot}78$ (Exp. 2; d.f. = 33) and $t = 1{\cdot}5$ (Exp. 3; d.f. = 54) were obtained. The difference for each experiment taken singly was insignificant, since on the 5 % level of significance, $t = 2{\cdot}03$ for d.f. 33 and $t = 2{\cdot}00$ for d.f. 54. A formula for extending the 't' test for multiple samples was used to combine the figures for the two experiments. This test showed the difference in growth to be above the 5 % level of significance.

In three further experiments the same comparison was made (Exps. 4–6), the results all tending to show that beriberi brain extract was less growth-promoting than normal brain extract. In Exps. 5 and 6 this difference was significant—'t' for Exp. 5 at 48 hr. being 5·15; at 96 hr. = 5·65 (d.f. = 70), and 't' for Exp. 6 at 48 hr. being 2·87; at 96 hr. = 3·7 (d.f. = 83). In Exp. 4 the difference was less. This may have depended on a wartime dietary deficiency in the food of the control pigeons, a defect that was remedied in the two subsequent experiments. The results indicate that extracts from the brains of pigeons suffering from beriberi are significantly less growth-promoting than are extracts from normal brains. The differences in relative increase of growth are illustrated in Figs. 2 and 3 based on the data of Exps. 5 and 6.

(3) *The influence of the addition of yeast or of aneurin to beriberi brain extracts.* In both experiments, three groups of cultures were used. Each experiment had control cultures fed on 5 % normal brain extract, cultures fed on 5 % beriberi brain extract, and cultures fed on 5 % beriberi brain extract to which yeast extract was added in the concentrations mentioned previously. In Exp. 4 a total of 78 cultures was used, in Exp. 5, 108 cultures.

A comparison of the growth of cultures fed on beriberi extract with those fed on beriberi extract with added yeast extract, showed an increased growth in the latter in both experiments. The difference in growth was significant at 96 hr. for each experiment taken separately. In Exp. 4 the value of t was 2·35 ('t' for significance at 5 % level = 2·00; d.f. = 54). In Exp. 5, $t = 2{\cdot}04$ ('t' for significance at 5 % level = 1·99; d.f. = 70). It can therefore be stated that some component of the B complex of vitamins is growth-promoting *in vitro*. It is unlikely to be biotin, which was lacking in the extract used. The growth curves of Exp. 5 are shown in Fig. 2.

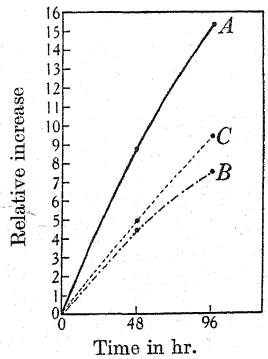

Fig. 2. Comparison of growth when yeast extract was added to media deficient in vitamin B complex. *A* = normal brain extract. *B* = beriberi brain extract. *C* = beriberi brain extract to which yeast extract was added.

In the next experiment aneurin chloride hydrochloride was added in the concentrations described earlier, instead of the yeast extract. A total of 129 cultures was used, divided into three groups. Group A was fed with a 5 % normal brain extract, group B with a 5 % beriberi brain extract and group C with a 5 % beriberi brain extract to which aneurin was added. Mean results showed no significant difference between the growth of cultures fed with beriberi extracts (15·72) and that obtained with beriberi extracts to which aneurin was added

(14·84). The growth obtained with normal brain extract was significantly greater than that in either of the experimental groups.

The values of 't' at the two periods of observation in comparisons of the three groups are given in Table 1.

Table 1. *Values of 't' obtained when the growth data for groups A, B and C (Exp. 6) are compared in pairs*

Comparison	Value of 't' at 48 hr. (Value of 't' for significance at 5 % level = 1·98)	Value of 't' at 96 hr.
A compared with B	2·87 d.f. = 84	3·7
A compared with C	2·6 d.f. = 84	4·34
B compared with C	0·36 d.f. = 87	0·607

The relative increase in outgrowth of the three groups is shown in Fig. 3.

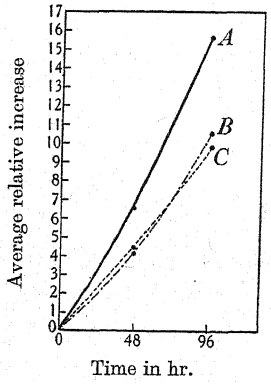

Fig. 3. Comparison of growth when additional vitamin B_1 was added to media deficient in vitamin B complex. A = normal brain extract. B = beriberi brain extract. C = beriberi brain extract to which vitamin B_1 was added.

The influence of normal chick-embryo extract containing added aneurin on the growth of fibroblasts in vitro

It has been shown in the previous experiments that vitamin B_1 acting by itself does not restore the growth-promoting capacity of beriberi brain, while the vitamin B complex does so, at least partially. The latter action may be due either to the presence of vitamin B_1 acting in concert with other members of the vitamin B complex, or it may be due to some factor in the vitamin B complex other than vitamin B_1. If the former explanation were true, it would be likely that vitamin B_1, in the presence of the normal embryonic extract and plasma, would improve the growth. Exp. 7 was designed to test this. A total of 54 cultures divided into two groups of 27 was used. Both groups were fed with equal volumes of 10 % extract from chick embryos, and plasma from normal birds. In addition, aneurin was added to one group in the same concentration as before.

In this experiment the growth obtained was almost equal in the two groups. The relative increase for the group without added vitamin B_1 was 4·86, that for the group with added vitamin B_1 was 4·49. The difference was insignificant since 't' = 0·41. At 5 % levels, $t = 2·008$ for d.f. = 53.

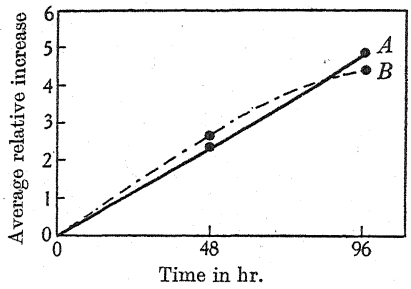

Fig. 4. Comparison of growth when additional vitamin B_1 was added to 10 % extract of normal chick embryos. A = normal embryo extract. B = normal embryo extract to which vitamin B_1 was added.

The graphs of the relative increases of growth (Fig. 4) compared with those in Fig. 3 showed that the types of growth curve obtained are similar in form whether aneurin is added to a normal medium, or to a medium obtained from beriberi birds. Within the technical limits of the experiment the members of each pair of curves can be regarded as identical.

DISCUSSION

Until now the effect of the B complex of vitamins on growth has largely been examined on animals *in vivo*. Experiments *in vitro* have the advantage of affording a more direct picture of the effect on cells grown in a standard substrate and they exclude to some extent influences which are indirect. The disadvantage of tissue culture experiments is the difficulty of defining and of measuring growth.

In the above experiments growth has been assessed as increase in area of the culture. It is appreciated that this 'growth' is a combination of cell division and outwandering. In order to ensure as far as possible that the ratio of division and outwandering for any experiment was a constant, the plasma coagulum was kept as constant as possible [Willmer, 1933]. All the plasma used for an entire experiment was obtained from one bird and the quantity used for each culture and the size of the spread drop were constant. The extracts used differed in their action, but except in Exp. 1 the dilution used in all sets of cultures in any one experiment was the same. It is therefore felt that in any experiment the mechanical properties of the medium and the accessibility of oxygen to the cells

were very similar. In Exp. 1 the dilutions of extract were of necessity different, but if the greater dilution induced more outwandering this would operate to lessen the significance of the results obtained.

In all the experiments in which extracts from normal brains were compared with extracts of brains from pigeons suffering from beriberi, it was shown that while growth occurred in the latter, it was less than that produced by brain extracts from normal birds. In beriberi it is very probable that there is a deficiency not only of aneurin, but of at least one other component of the B group of vitamins [Williams & Spies, 1939]. The reduced growth of cultures fed with beriberi brain extract might therefore be regarded as due to a deficiency of components of the vitamin B complex other than aneurin.

In examining the effect of the addition of vitamin B_1 or of the vitamin B complex, it had to be kept in mind that these substances may not necessarily act in the free state on cells. The results of those experiments, in which an extract of the vitamin B complex was added to media deficient in vitamin B, showed that increased growth could be produced in the presence of the media supplied. It is therefore presumed that any other factor necessary for the effect was present in the media. Since the extract of the vitamin B complex used was deficient in vitamin B_1 and in biotin, it is assumed that the growth-promoting substance was neither of these two.

In those experiments in which vitamin B_1 was added, no effect on growth could be shown. This was true whether or not the medium was obtained from animals judged to be lacking not only in those substances necessary for the formation of co-carboxylase, but also in other components of the vitamin B complex. No correlation could therefore be made between growth and the carbohydrate metabolism of the cell, and no effect could be shown whether vitamin B_1 was used alone or in the presence of the vitamin B of normal embryonic extract. The findings suggest that vitamin B_1 has no specific effect on cell growth as measured in these experiments. It would follow that the use of growth rate in animals as a measure of vitamin B_1 must be dependent on an indirect influence on the animal as a whole, as for example an effect on appetite.

It is felt that the methods described are satisfactory for further direct testing of the growth-promoting properties of constituents of the B complex of vitamins.

SUMMARY

1. An investigation was carried out to demonstrate the influence of the vitamin B complex and of vitamin B_1 (aneurin) on the growth of fibroblasts *in vitro*.

2. Extracts of the brains of pigeons suffering from beriberi were found to contain substances which promoted a satisfactory growth of fibroblasts. The growth obtained was, however, significantly less than that obtained when extracts of brains of normal pigeons were used.

3. The addition of yeast extract, which was deficient in vitamin B_1 and biotin, improved significantly the growth of fibroblasts which were grown in a medium deficient in vitamin B.

4. The addition of vitamin B_1 (aneurin) could not be shown to increase growth whether the medium was or was not deficient in its content of vitamin B complex.

5. From the data obtained, it is concluded that the vitamin B complex, or some component of it, has a specific effect on growth *in vitro*. This growth-promoting factor is probably not aneurin.

This work was carried out while one of the authors (E. P.) held the Pilkington Fellowship in Cancer Research of Manchester University.

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A Note on the possible Relationship between Ascorbic Acid and Glutathione *in vivo*

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In view of the fact that both glutathione and ascorbic acid belong to a redox system, it was thought that it might be of interest to determine the concentrations of both substances simultaneously in the same human subject during an extended 'saturation' test with ascorbic acid.

METHODS

Ascorbic acid in whole blood. Whole blood determinations were made by a slight modification of the method of Deeney, Murdock & Rogan [1942]. Pure CO was used for conversion of the haemoglobin to carboxyhaemoglobin. 2 ml. of blood were diluted with 4 ml. of glass distilled H_2O and 4 ml. of 16% HPO_3 used for precipitation. It was found impossible to remove all the suspended material by centrifugation on account of the presence of the octanol. After centrifuging, the solution was passed through a 5 cm. No. 41 Whatman filter paper which readily removed the large suspended particles.

Plasma ascorbic acid. 4 ml. of plasma were treated with 16 ml. of 3% HPO_3 solution as soon as possible after withdrawal of the blood. After centrifugation, 5 ml. portions of the clear supernatant fluid were titrated with a 0·01% solution of 2:6-dichlorophenolindophenol.

Glutathione in blood and plasma. The method of Woodward & Fry [1932] was employed. The analyses were carried out immediately after withdrawal of the blood and the appropriate corrections were made for the ascorbic acid content of the blood or plasma.

Two in-patients, W. (a male) and J. (a female) and one of us, V., were employed. No attempt at any kind of selection was made except that the third subject was chosen to give a range of initial plasma ascorbic acid values of 0·08, 0·26, 0·80 mg./100 ml.

A daily dose of 700 mg. of ascorbic acid for each 140 lb. of body weight was given as nearly as possible in two equal portions, one at the commencement of each 24 hr. collection of urine, the other approximately 12 hr. later. The dose was continued even when a 'state of saturation' had been reached. Blood samples were taken either at the end of the 24 hr. period, before the first dose of the subsequent 24 hr., or if no dose was given, at least 3 hr. after taking food. Blood samples in which the concentration of ascorbic acid was required were drawn into tubes containing 0·1 ml. of 20% potassium oxalate solution and 0·05 ml. of 5% KCN. The investigation was continued in subjects J. and V. after the administration of synthetic ascorbic acid had ceased for 3 and 7 days respectively.

Table 1. *Concentration of ascorbic acid and glutathione in the blood during and after an extended ascorbic acid saturation test*

Date	Ascorbic acid given orally mg./day	Ascorbic acid (mg./100 ml.) in		Reduced glutathione (mg./100 ml.) in			Oxidized glutathione (mg./100 ml.) in		Millimoles of	
		Whole blood	Blood plasma	Whole blood	Red cells	Blood plasma	Whole blood	Blood plasma	Ascorbic acid in blood plasma	Reduced glutathione in red cells
	W. aet. 55. Male. Haematocrit 27·1%. Wt. 120 lb. ?Cirrhosis of liver									
30. x. 42	0	—	0·08	37·1	137	2·5	4·0	0	0·0045	4·46
1. xi. 42	600	—	—	—	—	—	—	—	—	—
4. xi. 42	600	—	0·88	35·5	131	2·8	5·2	0	0·0500	4·27
9. xi. 42	600	—	1·08	31·3	116	2·8	0	8·6	0·0613	3·78
23. xi. 42	600	—	1·07	30·7	113	3·7	3·1	2·1	0·0608	3·68
	J. aet. 26. Female. Haematocrit 35·0%. Wt. 122 lb. Abdominal sinuses									
2. xi. 42	0	—	0·26	22·4	64	2·7	3·1	2·1	0·0147	2·08
10. xi. 42	600	—	—	—	—	—	—	—	—	—
11. xi. 42	600	—	0·98	22·4	64	1·5	4·9	5·5	0·0557	2·08
23. xi. 42	600	—	1·33	16·9	48	1·2	8·3	6·4	0·0756	1·56
27. xi. 42	0	—	—	—	—	—	—	—	—	—
30. xi. 42	0	—	1·12	18·4	53	0	0·9	6·1	0·0636	1·73
	V. aet. 40. Male. Haematocrit 46·0%. Wt. 158 lb. Normal									
23. xi. 42	0	0·82	0·80	35·0	76	0	4·6	4·4	0·045	2·48
24. xi. 42	750	—	—	—	—	—	—	—	—	—
25. xi. 42	750	1·53	1·53	30·1	65	0	7·6	1·5	0·087	2·12
30. xi. 42	750	1·48	1·48	28·8	63	0	3·1	3·4	0·084	2·05
1. xii. 42	0	—	—	—	—	—	—	—	—	—
7. xii. 42	0	1·08	1·02	31·9	69	0	2·5	5·2	0·058	2·25

RESULTS

The data (Table 1) show: (1) In all three examples the maximum ascorbic acid level is associated with a decreased concentration of glutathione in the red blood corpuscles, viz. 18, 24, 26% of the initial values in W., J., and V. respectively. (2) The plasma ascorbic acid content rises when the reduced glutathione in the red cells falls. Statistical analysis of the data by Mr H. T. Russell of the London School of Hygiene and Tropical Medicine showed that the values for plasma ascorbic acid and red cell reduced glutathione exhibited a negative correlation which is highly significant. (3) In the subject V., whole blood and plasma ascorbic acid varied equally in response to the test dose, although many authors find that the passage of ascorbic acid into the red blood corpuscle is slow [Borsook, Davenport, Jeffries & Warner, 1937].

DISCUSSION

Calculations from the data obtained show that the ratio of the fall in glutathione in the red blood corpuscles to the rise of the plasma ascorbic acid is approximately 10 mol. to 1 (variation 8–12). Further, in subjects J. and V. when the plasma ascorbic acid fell at the termination of the 'saturation' experiment, the rise in glutathione was of the same molecular order.

Possible relationships between ascorbic acid and glutathione and their interdependence as oxidation-reduction systems have been demonstrated by Hopkins & Morgan [1936] and by Crook & Hopkins [1938], who showed that reduced glutathione (GSH) prevented the oxidation of ascorbic acid *in vitro* even in the presence of its oxidase. Later Crook [1941], working with plant extracts, characterized a reductase which catalyzed the reduction of dehydro-ascorbic acid by GSH, and so was able to complete the system—plant substrate, dehydrogenase, GSH, reductase, dehydro-ascorbic acid and ascorbic acid oxidase—which allows the O_2 ultimately to bring about oxidation of the substrate. That dehydro-ascorbic acid is reduced in the body is well known [Johnson & Zilva, 1934], whilst Borsook *et al.* [1937] presented evidence that it was the GSH that was responsible for the reduction of dehydro-ascorbic acid in minced tissues. It is noteworthy that in order to obtain complete reduction a large molecular excess of GSH was required. Further evidence of the importance of the system in the body, and a suggested role in the formation of bile pigments, is that put forward by Lemberg, Legge & Lockwood [1941], autoxidation of oxyhaemoglobin to choleglobin occurring in the presence of ascorbic acid in turn reduced by GSH. Schultze, Harrer & King [1939] found no H_2 transfer to occur in a system consisting of nicotine haemochromogen, ascorbic acid, co-enzyme, and glucose with glucose dehydrogenase. Oxidation of ascorbic acid in the presence of Cu is inhibited by GSH [Barron, Barron & Klemperer, 1936; Mawson, 1935].

The relationship of the ascorbic acid and GSH levels in the blood established by us is additional evidence for the interplay of these systems. There are also indications that the fall in the GSH is accompanied by a rise in the oxidized glutathione (GSSG). It is tentatively suggested that one of the possible reasons why scurvy takes such a long 'latent period' to become manifest on a scorbutic diet is that in part the GSH in the tissues may tend to counterbalance the functional diminution consequent on the lack of ascorbic acid. The difficulty of ascribing a role to ascorbic acid in respiratory catalysis, on the grounds that tissues deficient in ascorbic acid fail to show decreased respiration [Elvehjem, 1942], may also, at least in part, be removed.

Our results for the glutathione content of whole blood are comparable with those of Woodward & Fry [1932] and of Platt [1931], although the highest values obtained for subject J. are rather below the lowest normal limit given by any of these authors (25 and 30·7 mg./100 ml. respectively). J. showed some degree of anaemia (Hb 56%), but the GSH content of the red cells is not in excess of that of V., a normal subject, a result which is not in agreement with the findings of Platt [1931] in anaemia, nor with our results in subject W. (Hb 52%).

SUMMARY

1. The concentration of glutathione in the red blood corpuscles of the human being varies inversely with that of the plasma ascorbic acid. Calculations show that the molecular change is approximately 10 to 1.

2. A possible role of glutathione in the long 'latent period' for scurvy is suggested.

We are indebted to Mr H. T. Russell of the London School of Hygiene and Tropical Medicine for help with the statistical analysis.

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Lysine Deficiency in Young Rats

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'Essential' amino-acids share with vitamins the property that they cannot be synthesized in the mammalian body and have therefore to be supplied in the diet. They differ from vitamins, however, in several other respects. Whilst vitamins are present only in small quantities in the tissues, amino-acids combined in proteins form a large part of the total mass of the body. The quantitative requirements for essential amino-acids are therefore considerably greater than those for vitamins. On the other hand, the body has a great store of amino-acids in its proteins, and the deleterious effects of a dietary deficiency of a particular essential amino-acid will therefore generally be less sudden and less marked, at least in the adult animal, than those of most vitamin deficiencies.

The clinical picture of a particular amino-acid deficiency will greatly depend on the biological function of the amino-acid concerned. If an amino-acid is needed only as building material for the synthesis of protein, a lack of that amino-acid in the diet will result only in an inhibition of protein synthesis and the effect will be more marked in the young animal than in the adult. The severity of the symptoms will depend on the quantitative requirements of the body relative to its stores of that amino-acid, and also on the extent of irreversible oxidation which occurs during the constant breaking-down and building-up of proteins in the mammalian body [Schoenheimer & Rittenberg, 1940]. This loss, which corresponds to the wear and tear quota of Rubner, may vary considerably for different amino-acids, and the effects of deficiencies on weight and nitrogen equilibrium may vary accordingly.

Some amino-acids, however, are apparently needed not only for the synthesis of proteins, but also for some specific metabolic purpose. For example, methionine acts as a donor of methyl groups and its absence from the diet produces specific symptoms peculiar to this amino-acid. Again, valine deficiency produces characteristic nervous symptoms [Rose & Eppstein, 1939] which suggest a special metabolic role for that amino-acid in the central nervous system. The deficiency or lack of such amino-acids will produce a clinical picture peculiar to the amino-acid concerned and will be superimposed on the general picture of deficiency common to all essential amino-acids which may be called a 'structural' deficiency.

This paper is concerned with lysine deficiency. Lysine was shown to be indispensable for the growth of young animals by Osborne & Mendel [1914; 1916], who also showed that animals could be kept alive for long periods and at fairly constant weight on diets containing very small amounts of lysine. Lack of lysine in young animals produces no specific symptoms apart from cessation of growth, and it has been claimed that it is not essential for maintenance in adults [Burroughs, Burroughs & Mitchell, 1940].

The animal kept at a constant weight by the lysine-deficient diet is, however, not static as regards the development of individual tissues and organs. Its body and tail lengths increase and so do the leg bones [Winters, Smith & Mendel, 1927], and many organs such as the kidneys and the eyes increase in weight, whilst others, like muscle, decrease [Lafon, 1939]. Male genital organs are not affected by lysine deficiency, but in females the

oestrous cycle is suspended [Pearson, 1937], and lactation is more affected than gestation [Hart, Nelson & Pitz, 1918; Fowler, Morris & Wright, 1934; Lafon, 1939]. It can be inferred from the absence of specific symptoms that lysine is required purely as passive material for the synthesis of proteins. This paper contains a description of the changes in the formed elements of the blood and in the blood proteins, and also of the changes in the processes of ossification, caused by a deficiency of lysine in the diet.

EXPERIMENTAL

Diets. The experimental animals, piebald rats of the laboratory stock (mostly males), were put on the diets soon after weaning and littermates were used as controls in each group. The main basal diet (diet A), which was given *ad lib.*, was as follows: gliadin 18%, arachis oil 10%, lard 5%, cod-liver oil 1%, salt mixture (McCollum) 5%, corn starch 61%. Basal diet B was identical with diet A except that gliadin was replaced by the same amount of zein, and a supplement of 10 mg. of *l*-tryptophan was always given daily. The water-soluble vitamins were given as a separate supplement in the following daily doses: aneurin 30 μg., pyridoxin 30 μg., riboflavin 50 μg., calcium-*d*-pantothenate 100 μg., nicotinic acid 1 mg., inositol 1 mg. and choline chloride 3 mg. The animals also received a daily dose of 0·5 ml. of a purified aqueous acetone extract of whole liver, of which 1 ml. corresponds to 4 g. fresh liver. This liver extract, which contained 11 mg. N/ml., gave a positive ninhydrin reaction and must be presumed to contain amino-acids.

The amino-acid supplements were fed with the vitamins. Adding the amino-acids to the basal diets did not affect the results. Lysine was given in the control experiments in the form of *l*-lysine hydrochloride, 40 mg./day.

RESULTS

Increase of total body weight

Table 1 shows that if young rats are reared on a diet in which practically all the N is derived from gliadin or zein, they stop growing and their body weight remains constant for long periods, confirming the old observations of Osborne & Mendel [1912, 1914]. Such a diet is by no means devoid of lysine. Osborne, van Slyke, Leavensworth & Vinograd [1915] isolated lysine from a gliadin hydrolysate and, after correcting for solubilities, arrived at a value of 0·64% for the lysine in gliadin. The indirect van Slyke distribution method indicates an even higher value and the lysine content can be tentatively put at about 0·9%. The liver concentrate included in our diet may also contain traces of lysine and the total daily intake of lysine on basal diet A may be as much as 10 mg.

Zein is believed to be completely free from lysine [Vickery, 1938] on the other hand, and basal diet B will contain only traces of lysine, from the liver extract and possibly from nitrogenous impurities in the starch. Since the results obtained with the gliadin and zein diets were almost identical, it can be concluded that the small amount of lysine in gliadin does not greatly affect the deficiency symptoms, at least in young rats.

If the basal diets are supplemented with sufficient lysine, growth occurs at a suboptimal rate (Table 1). We have found, in agreement with other workers, that an addition of 40 mg. lysine hydrochloride daily is sufficient to produce a maximal growth response with a gliadin diet. The increase in body weight, which varied from about 7–14 g./week (Table 1), is only about 40% of that produced by feeding a good protein, such as casein, at the same level. It appeared likely that gliadin might also be deficient in some other indispensable amino-acid. Rose [1938] found gliadin to be a poor source of threonine, and the value of 2·92% found by Winnick [1942] for the threonine content of gliadin is smaller than the values reported for other proteins such as casein or fibrin. An addition of 50 mg.

Table 1. *Total body weights of young rats receiving basal diets with or without added lysine*

Litter no.	Rat	Duration of exp. (weeks)	Basal diet	Lysine hydrochloride added daily (mg.)	Wt. (g.) Initial	Final	Increase/week
5	13	6	A	0	33	32	−0·17
	14	6	A	0	30	29	−0·17
	15	6	A	0	31	29	−0·33
	17	6	A	40	32	83	+8·5
	18	6	A	40	31	90	+9·8
6	1	7	A	0	32	33	+0·14
	2	7	A	0	30	33	+0·43
	3	7	A	0	30	29	−0·14
	4	7	A	40	28	71	+6·2
	5	7	A	40	30	73	+6·2
8	30	5	B	0	31	30	−0·20
	31	5	B	0	31	30	−0·20
	32	5	B	0	33	33	0·00
	33	5	B	40	32	67	+7·00
	34	5	B	40	32	69	+7·40

dl-threonine daily to a gliadin diet supplemented with lysine produced, however, no improved growth response. Likewise, addition of *l*-histidine, in which gliadin is also poor, did not affect the growth of the rats, nor was any improvement effected by addition of isoleucine or cystine to the diet. The suboptimal growth produced by supplemented gliadin diets is not readily explained by the amino-acid composition of this protein and must remain an open question at the moment. A similar lack of success in supplementing zein by different amino-acids has been recently reported by Borchers, Totter & Berg [1942].

If animals on the basal diet are compared with animals reared on a diet to which an optimal amount of lysine has been added, we observe in fact the differences produced by a diet low in lysine and a diet which contains sufficient lysine but is still, for an unexplained reason, incapable of producing optimal growth. Nevertheless, it appears reasonable to ascribe the differences observed between the animals reared on the basal diet and the controls to the insufficient quantity of lysine in the basal diet.

Red cells and haemoglobin

New-born rats have about 2·5–3·5 million red cells/cu.mm. and about 10 g. haemoglobin/100 ml. blood. At the age of 3–4 weeks, i.e. immediately after weaning, we find in our stock average values of 4,700,000 and 10·3 g. Hb respectively. On a casein diet containing all the vitamins these values rise during the following 2 months to about 8,000,000 red cells and 14 g. Hb. The changes which occur on a gliadin diet and a diet supplemented with lysine are shown in Table 2. All the figures are average values obtained from 10 to 12 rats in each group; the animals were about 3 weeks old at the beginning of the experiment. In the earlier experiments haemoglobin was estimated by the method of Herbert [1941], but in the later experiments we used the more accurate method of Rimington [1942].

Table 2. *Increase in number of red cells and haemoglobin in normal and lysine-deficient animals*

No. of weeks on diet	Red cells (millions/ml.)		Hb (g./100 ml.)	
	Normal	Lysine-deficient	Normal	Lysine-deficient
0	4·80 ±0·4	4·80 ±0·4	10·2 ±0·5	10·2 ±0·5
2	5·90 ±0·4	5·35 ±0·5	11·0 ±0·5	10·35 ±0·6
3	7·1 ±0·6	6·02 ±0·4	12·5 ±0·5	10·2 ±0·4
5	7·2 ±0·5	6·24 ±0·5	12·5 ±0·4	10·8 ±0·6
7	7·4 ±0·4	6·21 ±0·5	12·55 ±0·4	10·9 ±0·6

Table 2 shows that with a gliadin diet supplemented with lysine the rise of the cell count and of the haemoglobin content is almost normal. The values may be slightly lower than on a casein diet, but our data were not sufficiently numerous to establish this point with certainty. In the lysine-deficient animals both haemoglobin and red cells rise more slowly and the final values are lower by about 15–20 % than in the controls. This increase indicates that, although the total weight of the animals remains constant, the haematopoietic system continues to develop. The differences found between the lysine-deficient animals and the controls can hardly be interpreted in the sense that lysine deficiency produces an anaemia, but it may be inferred that the rate of development of the haematopoietic system is somewhat reduced by the lysine deficiency.

The effect of lysine deficiency on red cells is of particular interest in connexion with the anaemia produced by deaminized casein [Hogan & Ritchie, 1934], which can be cured by feeding relatively large quantities of lysine [Hogan, Powell & Guerrant, 1941]. Deamination destroys the terminal-NH_2 group of lysine and the resulting hydroxy or unsaturated compound is almost certainly unable to replace lysine in the diet. It appears, however, from the results reported here that the destruction of lysine in itself cannot account for the anaemia observed by Hogan. It must be assumed, in agreement with the conclusions reached by Hogan and his co-workers, that the treatment with nitrous acid produces a toxic substance either derived from lysine or from another amino-acid and that the curative effect of lysine is due to a specific detoxicating action of that amino-acid on the unknown toxic substances.

The conclusion that lysine deficiency does not cause anaemia is also supported by the results of Pearson, Elvehjem & Hart [1937], who found that recovery from nutritional anaemia produced by a milk diet is not greatly influenced by the quality of the protein in the diet during the recovery period. We have repeated their experiments with our gliadin and zein diets on three litters and have obtained results very similar to those of the American workers. It appears, therefore, that the relatively large amounts of lysine required for the formation of haemoglobin are taken from other tissues, if no dietary lysine is available.

Plasma proteins

At the end of the experimental periods the animals were killed and the blood was collected in vessels containing Na oxalate. There were no gross pathological changes in the experimental animals apart from a considerable wasting of muscular tissue in the lysine-deficient rats.

Plasma protein was estimated by multiplying the total N of plasma, as determined by the Kjeldahl method, by 6·25. In some cases the non-protein

nitrogen was estimated separately and deducted from the total N. This correction amounted to about 5–7 % of the total N; since the amount of plasma obtained was small, the determination of non-protein N was omitted in most experiments and a value of 35 mg. N/100 ml. plasma was deducted from the total N to give protein N. Table 3

Table 3. *Plasma protein concentration in normal and lysine-deficient animals*

Litter		No. of animals	Plasma protein (g./100 ml.)
1	Normal	2	5·5, 6·35
	Deficient	3	4·0, 4·3, 4·2
2	Normal	3	5·6, 4·95, 5·2
	Deficient	3	4·2, 4·2, 4·1
3	Normal	3	5·8, 5·6, 5·7
	Deficient	3	4·0, 4·2, 4·2
4	Normal	3	5·5, 5·9, 6·1
	Deficient	3	4·0, 3·9, 4·3

gives the results obtained with lysine-deficient and normal animals. Plasma protein values are considerably lower in the deficient animals; the first three litters had been reared on gliadin diets and the average plasma-protein value in the deficient animals is 4 % compared with one of 5·5 % in the controls. In litter 4, which had been on a zein diet, the plasma-protein values seem to be even lower. In two litters the albumin/globulin ratio was determined by the method of Howe [1921]. There was no significant difference between the lysine-deficient rats and the controls; in both groups the albumin/globulin ratio varied between 1·7 and 2·1.

Growth and ossification

The methods available for the assessment and comparison of rates of growth in animals, apart from mere weighing and measuring of bodily dimensions, depend on radiographic and histological studies. The former have the advantage that radiographs can be taken at any desired intervals during the course of the experiment; the latter have the disadvantage that they involve the sacrifice of the animal. The radiographic method gives a good idea not only of the rate of growth in the spine, tail, skull and long bones, but, with adequate 'soft tissue' technique, yields information about the bulk of the subcutaneous fat and muscular tissue. The histological method consists in recording the number of mitotic figures in the growing tissue or organ. In the case of the long bones, the actual number of cells in the columns of the zone of proliferating cartilage and the zone of calcifying cartilage in the epiphysial region may be counted. This method gives information not only of the rate of proliferation in a given type of cell, but also of the rate of differentiation such as occurs in osteogenesis and in the formation of bone marrow.

A comparison of the radiographs of the control and lysine-deficient rats at the age of 6 weeks, after 3 weeks on the diets, shows the great difference in bodily dimensions (Fig. 1, *a*, *b*). The normal animal is well covered with subcutaneous fat and muscle as compared with the starveling. The width of the epiphysial cartilage at the upper end of the tibia in the normal rat is approximately 1 mm., but that in the lysine-deficient rat is barely visible as a thin line, indicating that union of the epiphysis with the shaft is about to take place. The patella in the normal animal is a mass of bone nearly 3 by 1 mm., in the other it is a mere dot measuring less than 1 sq. mm. The caudal vertebrae in the lysine-deficient rat are smaller in all dimensions and the epiphysial plates are separated from the body of the vertebra by a narrower zone of epiphysial growth cartilage. The degree of calcification in all the bones is generally reduced as compared with the normal.

Two rats, one normal, the other lysine-deficient, aged 9 weeks, were killed after 6 weeks of feeding. Certain organs, including the wrist joint, knee joint and testes, were fixed in Allen's modification of Bouin's fluid at 38°, with a view to studying the minute anatomy of the epiphysial growth cartilage and the mitotic count of the testes. Sections were cut in paraffin and stained with haematoxylin and eosin.

The microscopic sections of the distal end of the radius (Fig. 2 *a*, *b*) show that in the normal rat the first zone of proliferating cartilage averages about 15 chondroblasts to the column as compared with about 7 in the lysine-deficient rat. The second zone of calcified cartilage averages from 3 to 5 cells in the column, but the trabeculae of calcified matrix are more clearly and heavily laid down in the lysine-deficient rat. The third zone of actual osteogenesis and bone marrow formation shows heavier transverse trabeculae of bone in the lysine-deficient rat, indicating arrest of growth. The bone marrow in these animals is composed largely of fat.

The microscopic sections at the upper end of the tibia similarly show in the first zone of columns of proliferating cartilage about 7 cells in the normal and 3 cells in the lysine-deficient rat, so that the evidence gained is definitely in favour of a marked arrest of growth in the lysine-deficient animal. In the case of the testes, the volume in the lysine-deficient animal was approximately 75 % of the normal and the number of mitotic figures in the tubules about 66 %.

It is clear from the comparison of the radiographs of the lysine-deficient rat at 3 weeks and 9 weeks of age, after 6 weeks of deficient feeding, that the internal economy of the animal has altered profoundly. Although the weight has remained constant, it is no longer the same animal. The subcu-

taneous fat has decreased, the muscles have wasted, the long bones have grown by about 20 % in length, but diminished in girth. The caudal vertebrae have increased by about 60 % in length but their diameter has slightly diminished.

DISCUSSION

The most striking effect of the deficient diet which contains only small amounts of lysine is complete cessation of growth without any loss of weight. It is possible that complete absence of lysine might result in a decrease of body weight.

Following the experimental technique of W. C. Rose and others, food was given *ad libitum*; the food intake of the deficient animals steadily diminished during the experiment and averaged during the last weeks only about 60–70 % of that of the controls. It may be argued, therefore, that the results observed are mainly due to the reduced calorie intake. The case for and against paired feeding, which eliminates differences caused by variations of food intake, has been reviewed at some length by Brody [1935]. It is certain, however, that the diminished appetite is the result of the deficiency of the diet and generally follows the failure of growth, as first observed by Hopkins [1912]. The diminished food intake is therefore indicative of a deficiency in the diet and it is immaterial for our purpose, i.e. the description of the symptoms of lysine deficiency, whether the effect is wholly direct or is largely caused by reduced intake of food, which in itself is due to the deficiency in lysine.

The constancy of weight in the deficient animals represents the sum total of changes in continuous operation. Certain organs such as the eye and kidney continue to grow at the expense of others [Lafon, 1939]. The long bones grow in length and the number of red cells/unit volume of blood increase. Whether the total number of red cells in the body and the total amount of haemoglobin change is impossible to say without measuring the blood volume. But growth, even where it occurs, is considerably retarded. This is shown very clearly by the histological analysis of the epiphysial bone and the reduced number of mitotic figures in the testes. Some tissues, such as muscle, lose weight, and it appears likely that the total protein of the body decreases slowly. A disturbance of protein metabolism is also indicated by the low serum protein values, which are reduced to oedema level. Protein appears to be transferred from some organs to others according to a definite system of priorities. The constancy of body weight shows that this transfer is done very economically, at least in lysine deficiency, with only a small loss of lysine through irreversible oxidation. The whole picture, which can best be interpreted as an inhibition of protein formation, is not unlike that produced by ordinary starvation, i.e. by reducing total intake of food [Jackson, 1908; 1932]. It is noteworthy, but not unexpected, that lack of one essential building material in the diet produces essentially the same symptoms as general underfeeding.

SUMMARY

1. A diet containing only small amounts of lysine produces, in young rats, cessation of growth and hypoproteinaemia. The number of red cells and the amount of haemoglobin/unit volume of blood are slightly lower than in the control animals which receive a diet supplemented with lysine and are growing. These low values are interpreted as indicating not so much an anaemia proper as a retarded development of the haematopoietic system.

2. Radiological examination shows a considerable decrease of subcutaneous fat, waste of muscle and reduction of calcification in the bones. The epiphysial cartilage in the long bones is barely visible and histological examination reveals a considerable reduction in the number of chondroblasts in the first zone of proliferating cartilage. In the zone of calcified cartilage the trabeculae of calcified matrix are heavier in the deficient animals than in the controls. Mitotic figures in the testes are reduced as compared with normal animals.

3. The changes observed are assumed to be due to a general inhibition of protein formation. This leads to a reduced growth of some organs which develop at the expense of others and protein is transferred according to a fixed system of growth priorities. The sum total of these changes is constant body weight. The similarity of this picture to that produced by starvation is discussed.

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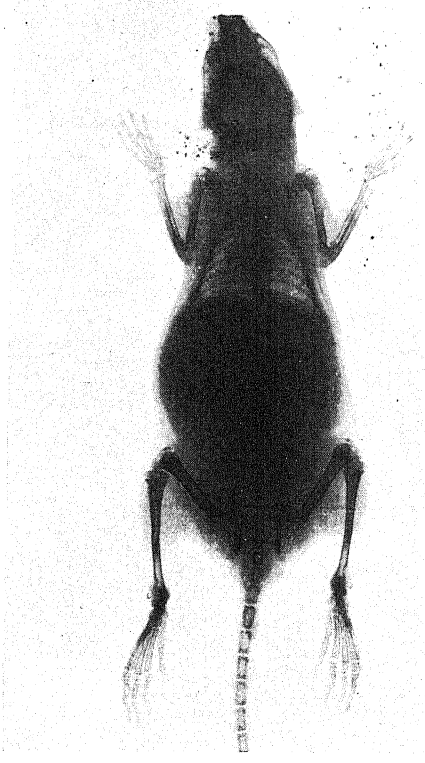

Fig. 1 *a*.

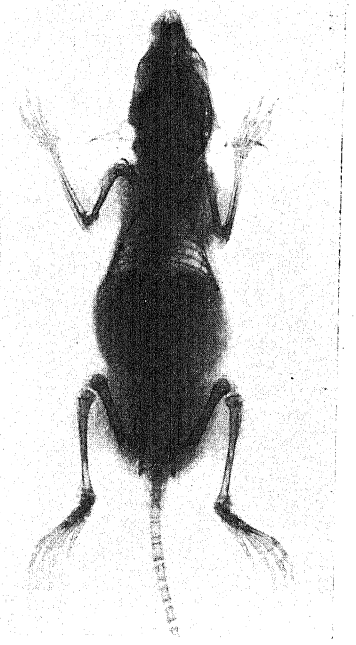

Fig. 1 *b*.

Fig. 1. Radiograph of lysine-deficient animal (1 *b*) and control (1 *a*). The animals were littermates, 10 weeks old and had been 6 weeks on their respective diets.

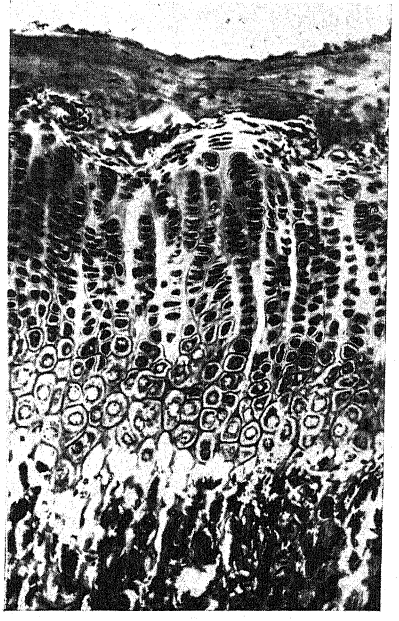

Fig. 2 *a*.

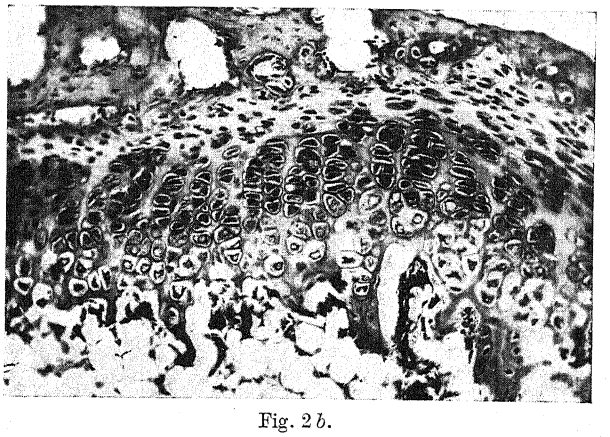

Fig. 2 *b*.

Fig. 2. Microscopic section of the distal end of the radius (*a*) of the normal, (*b*) of the deficient animal.

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The Mechanism of Coprosterol Formation *in vivo*

1. CHOLESTENONE AS AN INTERMEDIATE

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In view of the inherent improbability that the organism is able to convert cholesterol by direct hydrogenation into its *cis*-decalin derivative coprosterol, it has been suggested that the reaction proceeds in two stages [Rosenheim & Starling, 1933]. According to this view cholestenone, the intermediate oxidation product of cholesterol, is subsequently reduced in the intestine to coprosterol and partly to *epi*-coprosterol.

Although feeding experiments with cholestenone have since provided considerable experimental evidence for the correctness of this view [Rosenheim & Webster, 1935; Schoenheimer, Rittenberg & Graff, 1935; Rosenheim & Starling, 1937; Anchel & Schoenheimer, 1938], the final proof of the occurrence of cholestenone itself in the animal organism or in its excreta was still lacking. The difficulties of isolating such small amounts of cholestenone as escape complete reduction from the faeces of animals kept on an ordinary mixed diet, which is poor in cholesterol, are obvious. In our search for cholestenone we have, therefore, made use of the fact that brain contains a substance, the administration of which enables the organism to convert large amounts of cholesterol into coprosterol [Rosenheim & Webster, 1941]. By employing Girard and Sandulesco's ketone reagent, we have, under such conditions, been able to isolate cholestenone from the faeces of a dog and of rats fed on brain, and to characterize it both chemically and physically.

The isolation of cholestenone from faeces after feeding on brain necessitated a search for its possible occurrence in brain itself. The ultra-violet absorption spectra of cholesterol fractions obtained from various organs had been examined by Page & Menschick [1930 *a*, *b*; 1931], who were unable to find the characteristic absorption band of cholestenone at 2400A, in cholesterol prepared from normal brain. Their results do not, however, exclude the presence of traces of cholestenone which would have been removed by the solvents used in the preparation of the various cholesterol fractions. We therefore subjected brain to the treatment which led to the isolation of cholestenone from faeces. Although we obtained evidence that traces of a substance absorbing at 2380 A occur in brain, we were unable to characterize it as cholestenone. In view of its small amount, the possibility that this unidentified substance may be the source of the relatively large amount of cholestenone isolated from faeces after the administration of brain must be considered as remote.

Whilst this work was in progress, Marker, Wittbecker, Wagner & Turner [1942] succeeded in isolating *epi*-coprosterol from the faeces of a dog on a normal diet, thus bringing further support to the view that cholestenone is an intermediate in the formation of coprosterol in the organism.

EXPERIMENTAL

Cholestenone from rats' faeces. Rats of our Institute strain of Wistar rats and of the average weight of 200 g. were used. Six rats were kept on a diet of steamed sheep's brain and bone meal for 4 days, each rat consuming *ca.* 30 g./day of the diet. The housing of the animals, the preparation of the diet and the collection of the faeces have been described previously [Rosenheim & Webster, 1941].

The faeces of the last 3 days (140 g. moist) were collected, dried *in vacuo* over conc. H_2SO_4 and exhaustively extracted with ether in a Soxhlet. The lipids (23·5 g.) yielded on saponification 15·2 g. of unsaponifiable matter, which was dried in a high vacuum. 14 g. of the unsaponifiable fraction in acetic acid-ethanol solution were treated with 5 g. of the

'T' reagent of Girard & Sandulesco [1936]. The ketonic fraction, a yellowish oil weighing 0·4596 g., was dissolved in 25 ml. ethanol and its ultra-violet absorption examined. The absorption curve plotted from a 1:1000 dilution showed the characteristic absorption band of cholestenone with a well-defined maximum at 2400 A, the intensity of which, on comparison with a standard curve, indicated 0·144 g. cholestenone. The ketonic fraction, therefore, contained 31% and the unsaponifiable matter 1% cholestenone. In several similar experiments spectroscopic examination of the unsaponifiable fractions indicated variations of from 1 to 1·9% cholestenone.

The identity of the absorbing substance with cholestenone was established by the preparation of its well-crystallized *o*-tolylsemicarbazone [Rosenheim & Starling, 1937]. The solution of the ketonic fraction was mixed with a solution of 200 mg. of *o*-tolylsemicarbazide in ethanol containing 5 drops of acetic acid. After 24 hr. the semicarbazone had separated in hard, slightly yellowish aggregates of needles, weighing 0·2054 g., m.p. 238–239°, corresponding to 0·148 g. cholestenone. The m.p. rose to 240–241° after one recrystallization from butanol. A further recrystallization from dioxan gave colourless clusters of needles of cholestenone-*o*-tolylsemicarbazone, m.p. 242–243°, not depressed in admixture with an authentic specimen. Found: N, 7·9%. Calc. for $C_{35}H_{53}ON_3$: N, 8·0%.

Cholestenone from dogs' faeces. Our previous failure to induce coprosterol excretion in dogs fed on raw brain, whilst a diet of cooked brain, given to rats, raised the excretion of coprosterol to 80% of the faecal sterols [Rosenheim & Webster, 1941], led us to repeat the experiment with dogs fed on cooked, instead of on raw, brain. A male Airedale terrier, weighing 16 kg., under the care of our colleague, Dr G. L. Brown, received steamed sheep's brain mixed with 4% bone meal as his sole food for 4 days, and consumed *ca.* 350 g. brain/day. The faeces (310 g. moist) of the last 3 days, dried *in vacuo* and extracted with ether, yielded on saponification of the ether residue 25·2 g. of unsaponifiable matter as a semi-crystalline mass. On analysis by the gravimetric digitonin method, it was found to contain 16·4 g. sterols, of which 80·5% consisted of coprosterol. The cause of the strikingly different results obtained on feeding raw and cooked brain remains unexplained.

A solution of 20 g. of the unsaponifiable fraction was treated with the Girard-Sandulesco reagent as above described and yielded 0·810 g. of a ketonic fraction. The ultra-violet absorption curve of the latter in alcoholic solution showed a sharply defined maximum at 2400 A, indicating 0·203 g. cholestenone on comparison with a standard curve. The cholestenone content of the unsaponifiable fraction was, therefore, 1% and of the same order as in the case of the rats. The *o*-tolylsemicarbazone was prepared by the method above described and crystallized in colourless needles, m.p. 242–243°. Found: N, 8·2%. Calc. for $C_{35}H_{53}ON_3$: N, 8·0%.

Coprosterol from dogs' faeces. The non-ketonic fraction yielded 9·6 g. coprosterol, m.p. 98–100° on a single recrystallization from acetone-methanol solution. Coprosterol may be conveniently prepared directly from the faeces of dogs fed on cooked brain by mixing them with anhyd. $CaSO_4$ and extracting the dry product several times at room temperature, with acetone (cf. preparation of cholesterol from brain [Rosenheim, 1906]). The extracts are passed through a column of norit, freed from solvent and the colourless residue recrystallized from a mixture of acetone and methanol (2:1). This procedure, which is based on the fact that coprosterol occurs unesterified in faeces, yields a cleaner product than the usual method in which the lipidal impurities, introduced by the saponification of the primary ether extracts, complicate the purification of coprosterol.

Search for cholestenone in brain. 500 g. of minced ox brain were mixed with 1500 g. anhyd. $CaSO_4$. The hardened small lumps were extracted with 1200 ml. acetone at room temperature for 24 hr. and the extraction repeated 6 times. The extracts were taken to dryness *in vacuo* and the crystalline residue saponified in ethereal solution with an ethanolic solution of sodium ethoxide at room temperature. The ethereal solution, freed from soaps, was washed with dilute H_2SO_4 and H_2O. The crystalline residue (11·4 g.), obtained after removal of ether, was dried in a high vacuum and treated with the Girard-Sandulesco reagent as above described. Only traces of an oily ketonic fraction, weighing 28 mg., were obtained. Spectroscopic examination revealed a sharply defined absorption band with a maximum at 2380 A, the intensity of which, calculated as cholestenone, would indicate 7 mg. The identity of the absorbing substance with cholestenone, however, is doubtful since its characteristically insoluble *o*-tolylsemicarbazone could not be obtained. The suggestion, that the unidentified substance present in the calcified wall of the aorta and absorbing at the same wave-length [Page & Menschick, 1930*a*] may also occur in brain, may have to be considered. The fact that this substance is precipitable by digitonin [Schoenheimer, 1932] excludes its identity with cholestenone.

SUMMARY

The occurrence of cholestenone, characterized by its ultra-violet absorption and the preparation of cholestenone-*o*-tolylsemicarbazone, in the faeces of the dog and of the rat, after feeding on brain, has been demonstrated. The view that cholestenone is an intermediate in the conversion of cholesterol into coprosterol by the animal organism thus receives further confirmation.

We are indebted to Mr V. H. Parker for the ultra-violet absorption measurements and our thanks are due to Mr W. W. Starling for efficient assistance.

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The Availability of the Acetyl Derivatives of Lysine for Growth

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The biological behaviour of the amino-acid lysine is abnormal in several respects. In a number of experiments with isotopic nitrogen and hydrogen Schoenheimer and his collaborators [Foster, Schoenheimer & Rittenberg, 1939; Schoenheimer, Ratner & Rittenberg, 1939 *a*, *b*; Weissman & Schoenheimer, 1941] have shown that, unlike most amino-acids, lysine does not accept N from other nitrogenous compounds, nor could deuterium be introduced metabolically into this molecule. It appears that even the first stages of lysine metabolism are irreversible. Until now no substances other than *l*-lysine have been found that will support the growth of young rats on a lysine-deficient diet. Berg [1936] has shown that *d*-lysine, which does not occur in nature, cannot replace the natural *l*-isomer. The α-hydroxy analogue of lysine is equally ineffective [McGinty, Lewis & Marvel, 1924]. All other α-hydroxy analogues of essential amino-acids that have been studied, with the exception of that of cystine, can replace the corresponding amino-acid in the diet. Gordon [1939] has shown that neither α-dimethyl lysine nor α-monomethyl lysine will support growth on lysine-deficient diets. Neither of the mono-acetyl derivatives of lysine has previously been synthesized, and it was thought it would be of interest to see if they can be converted to lysine in the body.

Acetyl amino-acids are of especial interest in connexion with the theory of Knoop [1910], which assumes that they may be intermediates in the synthesis of amino-acids from α-keto acids. du Vigneaud, Cohn, Brown, Irish, Schoenheimer & Rittenberg [1939] have recently revived this theory and produced additional evidence in support of it.

The deacetylation of α-acetyl-amino-acids appears to be a fairly general reaction in the body. The α-N-acetyl derivatives of *l*-tryptophan [du Vigneaud, Sealock & van Etten, 1932], and *dl*-phenylalanine [Carter, Handler, Binkley, Fishback, Risser & Weisiger, 1938], have been shown to replace the respective amino-acid in the diet and du Vigneaud, Loring & Craft [1934] have shown that diacetyl- and diformyl-cystine are converted to cystine in the body. Similarly acetyl-*l*-homocystine [du Vigneaud, Dyer & Jones, 1937] and formyl-*l*-methionine [Jackson & Block, 1933] can replace methionine in the diet. The results of Kimura [1929] also suggest the presence, in most organs, of an enzyme capable of deacetylating amino-acids. It thus seemed likely that the α-acetyl lysine, at least, would be converted to lysine in the body; however, here again, it was found that lysine behaves differently from other amino-acids.

METHODS

Chemical

Preparation of acetyl lysines. For the synthesis of the α- and ε-acetyl derivatives of lysine, use was made of the fact that in the copper complex of lysine the α-NH_2 and the COOH groups are bound in a stable chelate structure to Cu, whereas the ε-NH_2 group is free and can react with acylating reagents [Kurtz, 1938]. ε-acetyl lysine was prepared by direct acetylation of the Cu complex. α-acetyl lysine was prepared through ε-carbobenzoxy lysine. The latter had already been prepared by Bergmann, Zervas & Ross [1935]. It can, however, be much more conveniently prepared in about the same yield by treating the Cu complex with carbobenzoxy chloride. The product is quite insoluble, and after treatment with H_2S, ε-carbobenzoxy lysine can be isolated. This is acetylated in the α position, and the carbobenzoxy group removed by catalytic hydrogenation.

(*a*) *ε-N-acetyl-l-lysine.* Excess (basic) $CuCO_3$ was added gradually to a boiling solution of *l*-lysine sulphate (1·9 g.). After cooling, the solution was filtered. 1 equiv. of $Ba(OH)_2$ (1·7 g.) was then added, and the solution cooled in ice. Then acetic anhydride (1·2 g.) and $Ba(OH)_2$ (2·2 g.) were added in portions over 30 min. with shaking and cooling. After standing for 30 min. at room temperature, 0·34 ml. (0·63 g.) H_2SO_4 was added, and H_2S passed through the solution. The $BaSO_4$ and CuS were filtered off, and well washed by resuspending in boiling H_2O. The filtrate and washings were combined and concentrated to about 20 ml. and excess of Ba^{++} or $SO_4^{=}$ removed. The solution was evaporated to dryness, the residue dissolved in a minimum of hot H_2O and absolute ethanol added till there was a faint cloud. On cooling, the acetyl lysine crystallized in the form of flat plates. It was filtered off and washed with aqueous ethanol, absolute ethanol and ether. Yield 0·6 g. Another 0·4 g. could be obtained by working up the mother liquors, making a total yield of 1·0 g. or 55% of the theoretical value. (m.p. 249–253° (decomp.). $(\alpha)_D = 3{\cdot}4° \pm 0{\cdot}2$ ($c = 4{\cdot}0$). Found: C, 50·9; H, 8·7; N, 14·6%. Calc. for $C_8H_{16}O_3N_2$: C, 51·0; H, 8·5; N, 14·9%. Amino N (van Slyke): found: 7·48. Calc.: 7·45%.)

It formed an insoluble derivative with phosphotungstic acid, but no insoluble picrate.

(*b*) *ε-N-carbobenzoxy-l-lysine.* 1·8 g. *l*-lysine monohydrochloride was treated with excess $CuCO_3$ as above. 5 ml. 2*N* NaOH was added, and the dark blue solution cooled in ice. Carbobenzoxy chloride (2 ml.) and 2*N* NaOH (10 ml.) were

added in ten portions over 30 min. with shaking and cooling, care being taken not to allow the solution to become too alkaline. The Cu complex, which separated as a blue precipitate leaving a colourless solution, was filtered off and washed well with H_2O and ethanol. It was suspended in 200 ml. of H_2O, and H_2S passed in with mechanical shaking. The solution was then brought to the boil and filtered hot. On cooling, the ϵ-carbobenzoxy lysine crystallized in fine needles. After standing in the refrigerator overnight it was filtered off; a little more could be obtained by evaporating the mother liquors to a small volume. Yield was 80% of the theoretical. A sample recrystallized from H_2O showed $(\alpha)_D = 14{\cdot}4°$ ($c = 1{\cdot}6$) in two equivalents of HCl. This agrees with the value 14·0° found by Bergmann *et al.* [1935].

(*c*) *ϵ-N-carbobenzoxy-α-N-acetyl-l-lysine.* 2·2 g. ϵ-N-carbobenzoxy *l*-lysine were dissolved in 10 ml. *N* NaOH and cooled in ice. 1·5 ml. acetic anhydride and 20 ml. *N* NaOH were then added in ten portions over 30 min. with shaking and cooling. After standing at room temperature for 20 min. the solution was neutralized with HCl, and extracted with ether. The ether layer was discarded, and the aqueous layer made acid to congo red. This precipitated the ϵ-carbobenzoxy-α-acetyl lysine as an oil, which was extracted with ethyl acetate. The extract was dried and evaporated to dryness, leaving an oil which was used directly for the preparation of α-acetyl lysine. To obtain the ϵ-carbobenzoxy α-acetyl lysine in a crystalline form, the ethyl acetate solution was extracted with dilute $NaHCO_3$. This was then acidified and the oil again extracted into ethyl acetate. After drying, the ethyl acetate solution was evaporated to dryness, and the resulting clear oil crystallized on standing overnight. It could be recrystallized by dissolving in ethanol and adding H_2O gradually. The product which crystallized slowly did not melt sharply; m.p. 105–115°.

(*d*) *α-N-acetyl-l-lysine.* The dry oil obtained from 2·2 g. ϵ-carbobenzoxy lysine was dissolved in ethanol, and a drop of acetic acid and a few ml. H_2O were added. It was then hydrogenated in the usual way. The α-acetyl lysine, which usually crystallized from the filtered and concentrated solution directly, was taken up in a small volume of H_2O, which was then concentrated in a desiccator till it started to crystallize. Absolute ethanol was added to complete the separation. The yield of pure α-acetyl lysine was 0·8 g., and a further 0·1 g. was obtained by adding ether to the mother liquors, which made a total of 0·9 g. or 62% of the theoretical value from ϵ-carbobenzoxy lysine. The overall yield from *l*-lysine was 46%. (m.p. 250° (decomp.). $(\alpha)_D = 4{\cdot}7°$ ($c = 4{\cdot}4$). Found: C, 50·4; H, 8·6; N, 14·5%. Calc. for $C_8H_{16}O_3N_2$: C, 51·0; H, 8·5; N, 14·9%.)

Biological

Animals. Young, recently weaned male rats of a piebald type were used. They weighed 30–50 g. at the beginning of the experiment. In each experiment three groups, each of two to three animals, were used, each group being constituted of the littermates of the animals in the other two groups. One group received the basal diet alone, another the basal diet supplemented by pure lysine, while the third group was given the basal diet together with the substance under test. The control groups were necessary because of the variation in growth response to the addition of supplements, from litter to litter.

Diet. The basal lysine-deficient diet, which was fed *ad lib.*, was constituted as follows:

Starch	61%	Lard	5%
Gliadin	18%	Cod-liver oil	1%
Arachis oil	10%	Salt mixture (McCollum)	5%

In addition, each animal received daily a mixture containing 30 μg. aneurin, 30 μg. pyridoxin, 50 μg. riboflavin, 100 μg. Ca-*d*-pantothenate, 1 mg. nicotinic acid, 1 mg. inositol, 3 mg. choline chloride, and 0·15 ml. of an aqueous concentrate of whole liver, corresponding to 2 g. fresh tissue.

RESULTS

Effect of oral administration of amino-acids

Rats fed on the basal diet alone failed to grow, but if 40 mg. *l*-lysine monohydrochloride were added to the daily ration the animals grew at the rate of 1–2 g./day, showing that lysine deficiency was the factor which limited growth. Addition of more than 40 mg./day of *l*-lysine monohydrochloride had no further effect on the growth rate.

The analytically pure acetyl lysines were fed at the rate of 42 mg./day, which is equivalent to a daily supplement of 40 mg. *l*-lysine monohydrochloride. The rats entirely failed to grow with oral α-acetyl lysine (Fig. 1), whereas with lysine they

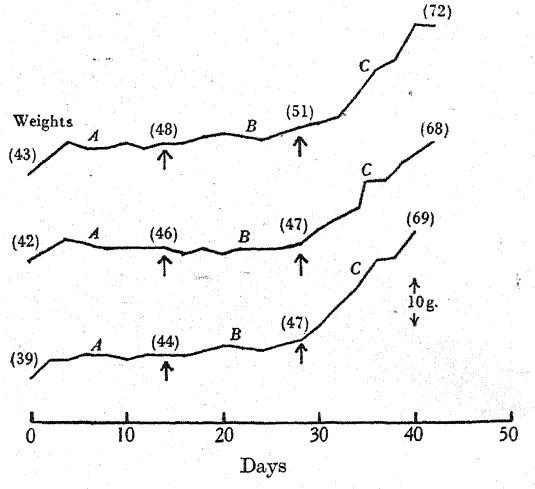

Fig. 1. The availability of α-N-acetyl-*l*-lysine for growth. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets fed to the animals were as follows: *A*, basal 18% gliadin diet; *B*, basal diet + 42 mg. α-N-acetyl-*l*-lysine/day; *C*, basal diet + 40 mg. *l*-lysine monohydrochloride/day.

grew about 1·5 g./day. This clearly shows that α-acetyl lysine cannot effectively replace lysine in the diet.

On the other hand, the growth rate with 42 mg. ϵ-acetyl lysine was almost as great as with lysine

(Fig. 2), and when 84 mg. were fed, the growth was equal to that with lysine; thus ε-acetyl lysine can be converted to lysine in the body to a considerable extent, although probably not quantitatively.

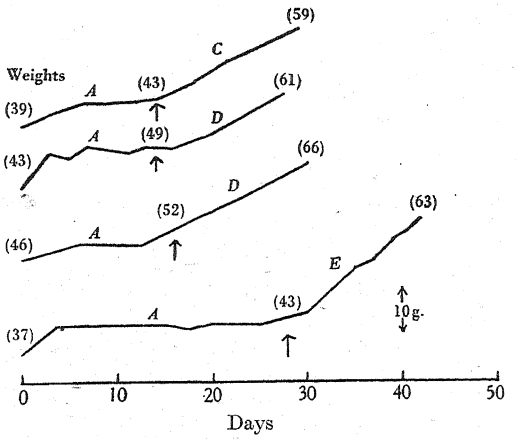

Fig. 2. The availability of ε-N-acetyl-*l*-lysine for growth. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets fed to the animals were as follows: *A*, basal 18% gliadin diet; *C*, basal diet + 50 mg. *l*-lysine monohydrochloride/day; *D*, basal diet + 42 mg. ε-N-acetyl-*l*-lysine/day; *E*, basal diet + 84 mg. ε-N-acetyl-*l*-lysine/day.

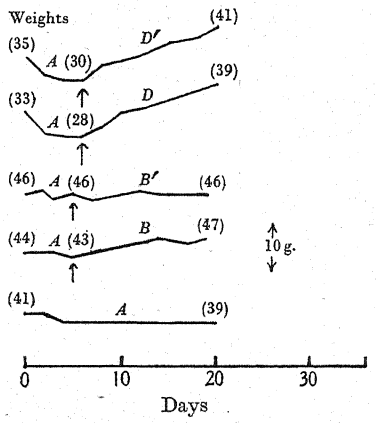

Fig. 3. The effect of intraperitoneal injection of the acetyl lysines. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets were as follows: *A*, basal 18% gliadin diet; *B*, basal diet + 42 mg. α-N-acetyl-*l*-lysine (fed)/day; *B′*, basal diet + 21 mg. α-N-acetyl-*l*-lysine (injected twice daily); *D*, basal diet + 42 mg. ε-N-acetyl-*l*-lysine (fed)/day; *D′*, basal diet + 21 mg. ε-N-acetyl-*l*-lysine (injected twice daily).

Effect of parenteral administration of amino-acids

It appeared possible that the reason why α-acetyl lysine did not effectively replace lysine was its failure to be absorbed from the gut. Furthermore, ε-acetyl lysine may have been able to replace lysine because it was hydrolyzed to lysine by bacteria in the intestine. The acetyl lysines were therefore injected intraperitoneally into rats, any changes that may take place in the gut thus being avoided. Conrad & Berg [1937] have shown that when lysine is fed in one dose each day it gives the same growth response as when it is fed in half the dose twice daily; they also showed that better results are obtained if the daily dose is given in two injections at 12 hr. intervals. In our experiments the rats fed on the basal diet were given two daily injections of a solution containing 21 mg. acetyl lysine. The results (Fig. 3) confirm those of the feeding experiments, and thus show that the differences then observed were not due to any reaction which may take place in the gut.

DISCUSSION

The feeding experiments demonstrate that for the young growing rat, ε-acetyl lysine can replace *l*-lysine in the diet; this is presumably due to its conversion into lysine by deacetylation to give its parent amino-acid. α-Acetyl-*l*-lysine, however, is not appreciably converted. This may be due to its not being broken down at all. This seems unlikely since other α-N-acetyl-amino-acids can be metabolized very readily. A more likely explanation is that it is oxidized, presumably at the ε-NH_2, before deacetylation can take place, whereas with the ε-acetyl compound deacetylation takes place more readily than oxidation of the α-NH_2. If we assume that the rate of deacetylation is about the same in both cases, then it would appear that the ε-NH_2 is more readily oxidized than the α-NH_2, and this may be true for lysine itself, which suggests that the initial breakdown of lysine may take place at the ε-NH_2 and not at the α-NH_2 group. However, it is probably unwise to compare the metabolism of the acetyl-lysines, in which one NH_2 is masked, with that of lysine itself, since the action of enzymes will probably be affected by the presence of additional free NH_2 groups.

The results with α-acetyl lysine are of interest in connexion with the acetylation theory of Knoop [1910]. Since α-acetyl lysine, the assumed intermediate in lysine synthesis, cannot be converted into lysine, this would explain the fact that lysine cannot be synthesized in the body from the corresponding hydroxy or keto-acid, and that N^{15} cannot enter the α-NH_2 of lysine. However, it was not suggested by Knoop, and it seems unlikely, that acetylation combined with reductive amination is the only method of amino-acid synthesis; but our results do seem to lend support to the theory.

SUMMARY

1. Methods are described for the synthesis of ε-carbobenzoxy lysine, α-N-acetyl lysine and ε-N-acetyl lysine from the copper complex of lysine.

2. ε-N-acetyl-*l*-lysine is available for the growth of rats on a lysine deficient diet, whereas α-N-acetyl-*l*-lysine is not so available.

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The Thermal Decomposition of Aneurin and Co-carboxylase at Varying Hydrogen Ion Concentrations

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Molitor & Sampson [1936] have stated that pure aneurin (vitamin B_1 hydrochloride) in aqueous solution at *p*H 3·5 may be heated to 120° without undergoing decomposition. Foodstuffs containing aneurin are rarely if ever prepared, cooked and eaten in such an acid condition, and this observation, therefore, is nutritionally of little practical importance. Farrer [1941] has made an extensive study of the effect of *p*H's ranging from 3 to 9 upon the destruction of aneurin in solution at 100° and finds that even at *p*H 3 there is 16 % loss after 1 hr. and 29 % after 3 hr. It may therefore be that assay methods were not sufficiently advanced at the time of publication of Molitor & Sampson's work to indicate with certainty the small loss which it would appear probable had taken place. On the other hand, as will be discussed more fully later, the buffer used by Farrer may have contained impurities which catalyzed the destruction which was noted. Beadle, Greenwood & Kraybill [1943] have verbally presented details of a study similar to that of Farrer, but a full account of their work has only just come to hand.

Apart from these references to rate of destruction under specific and carefully controlled conditions and certain early studies [Chick & Hume, 1917; Sherman & Grose, 1923; Sherman & Burton, 1926; Guha & Drummond, 1929] there is little factual information in the literature on the subject apart from statements concerning losses on baking bread and in cooking a few other foodstuffs. In view of the fact that the fortification of foodstuffs with vitamins and other dietary essentials is being widely carried out (in respect of vitamin B_1 and other factors) it was considered desirable to determine such losses over a fairly wide range of conditions (but in the absence of oxidizing or reducing agents) which would serve to indicate the magnitude of loss to be expected in any non-pressure cooking or preparatory process (e.g. blanching) applied to foodstuffs.

While wheat and some other cereals contain vitamin B_1 as hydrochloride only, in other vegetable (e.g. soya) and most animal products B_1 activity resides in the pyro-phosphoric ester (co-carboxylase) and/or monophosphate of the vitamin in addition

to the free non-esterified form. Also, when a fermentation process is applied to a foodstuff (e.g. panary fermentation), phosphorylation of its vitamin B_1 takes place. In view of the uncertainty as to the relative stabilities of free aneurin and co-carboxylase it was decided to compare them under identical conditions. Clearly, if there is any foundation for the suggestion of Rosenberg [1942] that the relatively high stability of vitamin B_1 in foods as measured by activity is due to the vitamin being present largely in this combined form, then, other things being equal, fortification of foods should preferably be carried out with co-carboxylase rather than with aneurin.

The effect of metallic ions (especially Cu^{++}) in catalyzing oxidation was also considered as a possible factor in the destruction of aneurin. There is no doubt that many of the more labile constituents of foodstuffs may be profoundly affected during their cooking by the nature of the vessel in which the cooking is carried out. Therefore the effects of a number of metals of which cooking vessels are usually constructed or with which they are lined were compared at concentrations low enough to be of the same order as those which might be expected to occur in practice. Metals used in this connexion were Al, Zn, Sn, Fe, and Cu.

EXPERIMENTAL

Aneurin destruction. Glass distilled water was used throughout. Buffer solutions were made from Analar reagents as follows: 0·2*M* acid potassium phthalate; 0·2*M* acid potassium phosphate; and a solution 0·2*M* in respect of both of the above salts. This latter mixed buffer was accurately calibrated for addition of *N*/5 acid and alkali using an electrometric *p*H meter (quinhydrone electrode) accurate to 0·02 *p*H, and was used to cover a range of *p*H 5·4–6·8. The accuracy of buffers made from phthalate and phosphate separately (after the method of Clark & Lubs) was also regularly checked on the same meter.

A solution of pure aneurin was prepared in glass distilled H_2O (previously acidified to *N*/25 with HCl) to a final strength of 40 μg./ml. Such solutions have been found to be quite stable over periods of many months.

Solutions of the following salts were prepared containing 80 parts/million of the metal in each instance: $CuSO_4$, $ZnSO_4$, $FeCl_3$, $FeSO_4$, $SnCl_2$, $Al_2(SO_4)_3$.

Rate of destruction of aneurin was studied at *p*H's ranging from 2·4 to 7·4, particular emphasis being laid on the *p*H 5–7 range in view of the relative rarity of foods falling outside this range.

50 ml. of the appropriate buffer solution was taken in a 250 ml. Erlenmeyer flask and was adjusted to the desired *p*H with *N*/5 HCl or NaOH. In all instances insufficient *N*/5 HCl was used by exactly 1 ml. (or excess *N*/5 NaOH by 1 ml.) in order to allow for the subsequent addition of 5 ml. of the *N*/25 acid aneurin solution. In appropriate cases, 5 ml. of the metal solution were added, and then in all instances the volume of solution was made up to 195 ml. with H_2O. One or two minute fragments of porous pot were added and the solution was quickly brought to boiling point, 5 ml. of the aneurin solution (containing 200 μg., i.e. giving a final concentration of 1 μg./ml.) were added and boiling of the solution was started immediately under reflux, an all-glass system being used.

The time at which the aneurin addition was completed was noted and the contents of the flasks were sampled at the times indicated on the graphs of rate of destruction (Figs. 1–3)—usually after $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2 and 3 hr. Sampling was carried out by momentarily removing the source of heat from the flask, and then rapidly pipetting 20 ml. of the solution into a tube containing 1 ml. of conc. HCl immersed in cold H_2O. By this means, destruction of the aneurin was immediately stopped. The final solution for assay even in the absence of destruction, would contain slightly less than 1 μg./ml., and this amount was determined both theoretically and experimentally to serve as a 'control' value and basis for assessing the percentage destruction which had occurred. Experimentally it was determined by rapidly bringing an acid solution of aneurin (*p*H 3, conc. 1 μg./ml.) to the boil and immediately pipetting 20 ml. into 1 ml. of conc. HCl. Theoretical and experimental values agreed within the limits of experimental error. At the conclusion of the 3 hr. boiling, all solutions after cooling were checked for *p*H. In certain instances, buffers of double the above concentration were used. Rates of destruction of aneurin in these cases were identical with those obtaining with the lesser concentration and are therefore neither graphed nor considered separately.

Co-carboxylase destruction. Co-carboxylase (aneurin pyrophosphate) cannot be estimated by the thiochrome method until it has been split up into phosphoric acid and aneurin. The plan of the experimental work had therefore to be substantially altered to make possible the complete hydrolysis of the ester before assay was carried out.

All estimations of co-carboxylase destruction were made using the mixed buffer. The co-carboxylase used was supplied by Merck and Co., Inc. (U.S.A.) and a solution containing 800 μg./5 ml. in *N*/25 HCl was prepared. The stability of this solution at room temperature was equal to that of aneurin solution under similar conditions—there was no loss after several months. In view of the fact that the co-carboxylase in this solution would become converted to monophosphate within a short time, experiments were carried out on old and freshly prepared solutions, i.e. on monophosphate and co-carboxylase, but no difference was found in their behaviour. It was therefore not considered necessary to use fresh co-carboxylase solutions for the work.

The setting up of the flasks was substantially the same as for aneurin destruction experiments—40 ml. of buffer solution + the necessary amount of *N*/5 NaOH to give the desired *p*H (after the addition of the 5 ml. of *N*/25 acid co-carboxylase solution) + H_2O to bring the total volume to 155 ml. The 5 ml. of co-carboxylase solution were added in a similar way to that described for aneurin, and 20 ml. portions were removed from the total volume of 160 ml., also in the same way as for aneurin. (Each 20 ml. of solution, it should be noted, originally contained 100 μg. of co-carboxylase.) Each portion was run into a 100 ml. flask containing sufficient *N*/5 HCl to bring the *p*H of the contents to $4 \pm 0{\cdot}1$, was diluted to approx. 50 ml. and as soon as its temperature had fallen to about 40°, 1 ml. of a solution of taka-diastase in 15% ethanol (representing 50 mg. of the Parke Davis standard dry substance) was added. Incubation at 37° overnight (16 hr.) brought about complete hydrolysis of the co-carboxylase; after coming to room temperature the contents of each flask were made up to 100 ml. and was assayed.

Vitamin B_1 assay. Vitamin B_1 assay was carried out by a photoelectric procedure substantially the same as has been detailed elsewhere [Booth, Nicholls, Kent-Jones & Ward, 1942]. This method has been shown [Coward, 1943] to have limits of error for pure solutions of aneurin of the concentrations encountered here as follows:

No. of estimations	$P=0{\cdot}95$	$P=0{\cdot}99$
In duplicate	1·98 %	2·60 %
In triplicate	1·62 %	2·12 %
In quadruplicate	1·40 %	1·84 %

In a number of instances the boiled buffer + aneurin solutions containing aneurin decomposition products were exposed to ultra-violet light in order to ascertain whether thiochrome was formed during the boiling, but in no instance was this the case nor was any fluorescence observed. The same was true of isobutanol extracts from these solutions. It was therefore considered unnecessary to carry out unoxidized 'control' estimations to any considerable extent, as the 'control' values were both very low and uniform.

Each point on the curves represents the average of at least six separate determinations. Replicates agreed very well; only occasionally a phenomenally high rate of destruction was noted. This so far unexplained circumstance has been observed also by Dawson [1943]. Despite all reasonable precautions being taken, an occasional flask showed marked acceleration of destruction, this not uncommonly being at double the rate normally observed. It was considered that this might possibly be due to dissolved O_2, but bubbling N_2 through the solution for 20 min. before carrying out an experiment did not prevent the occurrence of occasional freak destruction rates. Once brisk ebullition has commenced with consequent rapid loss of dissolved gases the risk of oxidation due to dissolved O_2 rapidly diminishes [Mapson, 1941]. Therefore as a further check the buffer solution was boiled under reflux for ½ hr. before adding a solution of aneurin made up in previously boiled $N/25$ acid, the experiment being otherwise continued in the usual manner. Again, the absence of O_2 from the solution had no effect upon destruction rate.

RESULTS

In addition to the data presented graphically (Figs. 1–4), further figures were obtained which are not inserted on the graphs in order to avoid confusion.

Within the limits of experimental error, no effect was observed upon the rate of destruction of aneurin at *p*H 5·4 in mixed $M/20$ phosphate-phthalate buffer by the addition of 2 parts/million of Zn, Fe^{++}, Fe^{+++}, Sn and Al, but Cu caused it to be markedly accelerated. In view of the greater rate of destruction of co-carboxylase as compared with aneurin under identical conditions, it was considered possible that the co-carboxylase contained an impurity which catalyzed destruction. Addition of co-carboxylase to aneurin solution at *p*H 5·4 at the rate of 1γ of co-carboxylase/1γ of aneurin/1 ml., however, failed to accelerate the destruction of the aneurin. The removal of O_2 from the buffer solutions (prior to addition of aneurin solution), either by bubbling of N_2 for 20 min. or by boiling under reflux for ½ hr., affected neither the rate of the destruction at *p*H 5·4 during ½ hr. (when under the particular experimental conditions the maximum effect would be expected), nor the total amount of destruction during 3 hr. boiling.

DISCUSSION

It is clear both from the shape of the graphs and also from the evidence in respect of dissolved gases that primarily the destruction of aneurin at 100° is thermal and is affected by *p*H; it is not an oxidative effect. The stability of aneurin in solution at room temperature at low *p*H's where no protection is given against atmospheric oxidation also supports this view. Nevertheless, as Cu is able to catalyze destruction, it would appear that oxidation can be involved. From the point of view of food processing, therefore, minimal loss of aneurin is achieved by the employment of as low a *p*H as is practicable coupled with the avoidance of contact with Cu and with the minimum exposure to high temperature during cooking.

It will be noted that where their respective *p*H ranges overlap, there is a considerable difference between rate of destruction in phosphate and phthalate buffer (compare Figs. 1 and 2). When Fig. 3 (mixed buffer) enters into the comparison it becomes clear that phosphate exerts a protective effect of some kind, as destruction in the presence of phthalate is lessened by the simultaneous presence of phosphate.

On comparing the relative rates of destruction of co-carboxylase and aneurin under identical conditions, it is evident that the former is very much less stable than the latter. The reason for this is obscure, but it is quite definite and unmistakable and disposes of the hypothesis of Rosenberg [1942] previously mentioned. It furthermore rules out the desirability on other grounds of using co-carboxylase in lieu of aneurin for fortifying foodstuffs.

It will be noticed that Farrer [1941] obtains destruction rates (using citrate, phosphate and biborate buffers) in most cases roughly double those reported here. He also mentions that his figures 'show that aneurin is considerably more vulnerable to heat than results obtained by means of animal experiments would suggest'. While the figures reported here are still somewhat in excess of those reported by Sherman & Burton [1926], concordance is very much greater. It must also be borne in mind that Sherman & Burton's figures were obtained at a time when biological assays were not susceptible of the accuracy obtainable at present and in fact at a time when differentiation of the B group of vitamins had not gone very far.

The shape of the curves in Fig. 2 is worthy of comment—so far no reasonable explanation has

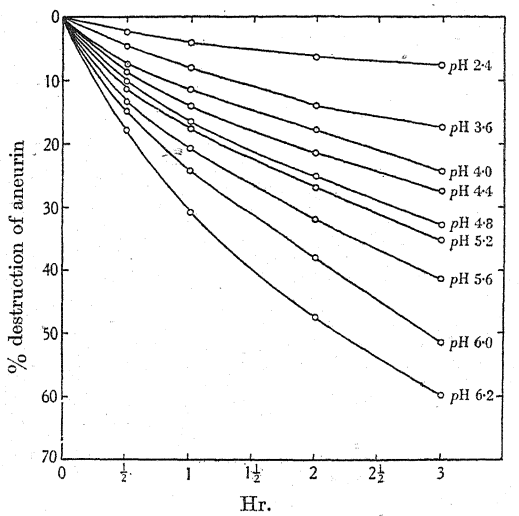

Fig. 1. Destruction of aneurin in boiling phthalate buffer.

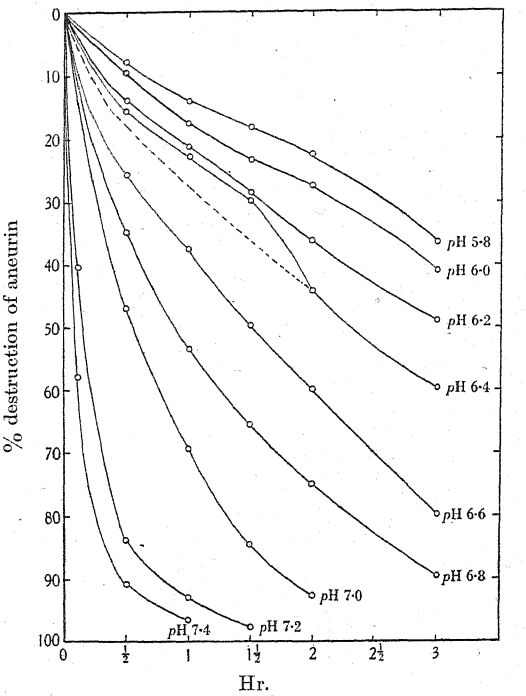

Fig. 2. Destruction of aneurin in boiling phosphate buffer.

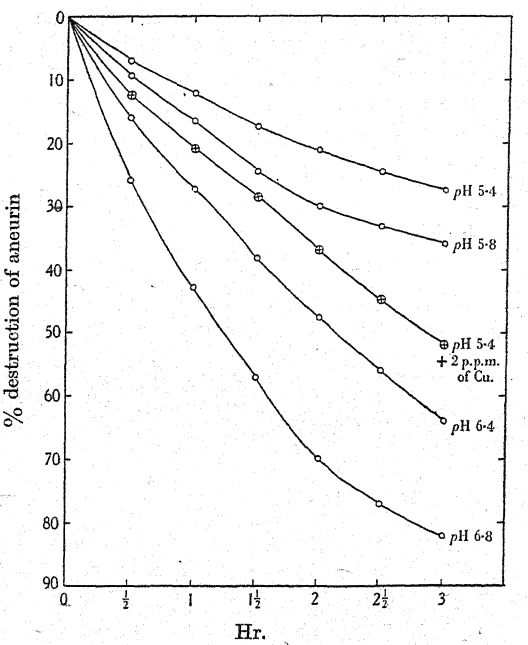

Fig. 3. Destruction of aneurin in boiling mixed (phosphate + phthalate) buffer, with and without 2 p.p.m. Cu.

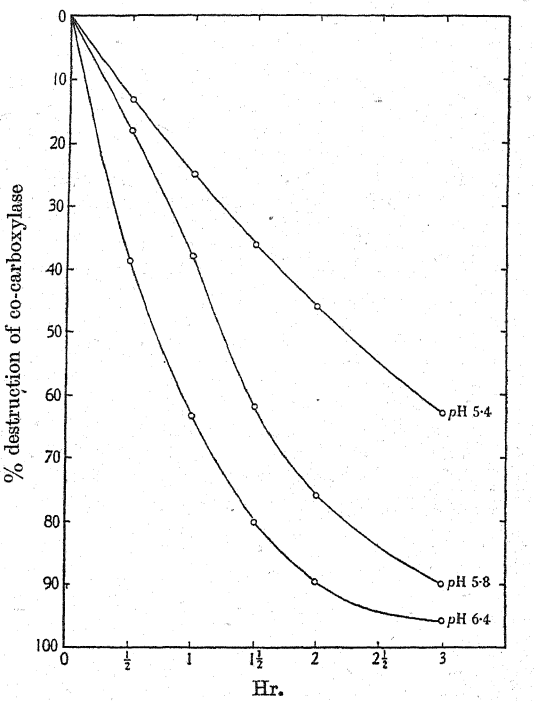

Fig. 4. Destruction of co-carboxylase in boiling mixed (phosphate + phthalate) buffer.

been found for the pronounced sigmoidal tendency at *p*H's 5·8 and 6·0, nor for the peculiar 'step' in the 6·4 curve.

While it is evident that to apply the figures presented here, obtained under rather artificial conditions, to foodstuffs in general would be unwarrantable, nevertheless there is evidence that they give a reasonable indication of the order of loss to be expected. For example, Moir [1942] finds a loss of 5–10 % of aneurin in oatmeal on boiling for 30 min., while he reports the *p*H of oatmeal to approximate closely to 6. Slater & Rial [1942] also find a small amount of destruction in domestic cooking of porridge. A rough average of the figures indicated by Figs. 1–3 gives an expected loss of about 12 %—if speculation may be allowed, the possibility of the antioxidant in oats reducing destruction, and/or the fact that porridge is not briskly boiled and may therefore fall short of 100° might be considered. Dawson & Martin [1941] find a loss of 8 % of vitamin B_1 in breadmaking, Hoffman, Schweitzer & Dalby [1940] find 5–9 %, Aughey & Daniel [1940] find 14 % loss, while Schulz, Atkin & Frey [1942] place baking loss at approximately 20 %, which at a *p*H of approximately 5·7–5·8 again agree well with the order of loss indicated here.

Aughey & Daniel [1940] also studied aneurin losses on cooking vegetables and determined *p*H's of the cooking liquors in some cases. While such *p*H's may not adequately represent the *p*H of the foodstuff cooked, significant support is given for the validity of the figures in Figs. 1–3 by *p*H and heat losses on cooking peas (9 % loss on simmering 12 min. at *p*H 6·4) and snap beans (18 % loss on simmering 40 min. at *p*H 5·8). A peculiar feature of their series of results is the rather inexplicable absence of destruction of aneurin in navy (dried) beans cooked for almost 1½ hr.

The increase in destruction of aneurin in foods occasioned by the addition of sodium bicarbonate is well known (e.g. Aughey & Daniel [1940]; Fincke [1941]; Barackman [1942]) and can be completely accounted for by the *p*H shift which it causes.

SUMMARY

1. The destruction rates of aneurin and co-carboxylase in various buffers at 100° have been followed by means of the thiochrome method of estimation.

2. The effect of traces of various metals and of atmospheric oxygen upon the destruction rate have also been compared.

3. Co-carboxylase is very much more thermolabile than aneurin at the same *p*H.

4. While Cu at the rate of 2 parts/million catalyzes the destruction of aneurin, Fe, Al, Zn, and Sn do not.

5. Destruction of aneurin appears to be for the most part non-oxidative in nature.

6. Comparison of the figures obtained in (1) with published figures by other authors of losses on cooking various foodstuffs shows reasonably good agreement.

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Vitamin E Deficiency in the Rat

V. UTERINE CHANGES IN CHRONIC DEFICIENCY

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In a previous paper [Barrie, 1938*b*] I described the degenerative pigmentation of the uterus produced in the rat by chronic vitamin E deficiency, which was first observed by Martin & Moore [1936, 1938]. It seemed important to find out whether or not this condition could be cured, at any stage of its development, by administration of vitamin E, and biopsies of the uterus at various stages before and during treatment have therefore been carried out.

EXPERIMENTAL

Laparotomy was performed under ether anaesthesia and a small portion of the left horn of the uterus was removed. This entailed removal of the left ovary. In one case, rat 1081, the ovary was implanted in the peritoneum. The whole operation was carried out under strictly aseptic conditions, the wound being kept as small as possible.

Twenty-nine vitamin E-deficient rats were chosen, all of which had received from the time of weaning the same vitamin E-free diet, E5 [Barrie, 1938*a*], but for varying periods ranging up to 10 months. The animals received daily doses of a vitamin E concentrate or synthetic *dl*-α-tocopherol for a period of approximately 2 weeks following operation. In 18 of the rats the procedure was then repeated, the old wound being reopened and another piece of the uterus being removed; a third operation was performed on two of these animals. There was no sepsis and no deaths in any of the 49 operations.

After further treatment with vitamin E, the animals were mated and then killed either at the end of the resorption, or after the litter had been weaned. The last dose of vitamin E was always given on the day of mating.

One of the operated rats, 1081, did not become pregnant in spite of five matings with different males when it was on full oestrus. The animal was killed and a post-mortem examination was made, but no abnormality other than the uterine discoloration was found. Another animal, 1541, was exceptional in that it never came on oestrus following operation; anoestrous smears were obtained on 73 consecutive days, after which it was killed and post-mortem examination revealed a cyst on the remaining ovary.

The size and colour of the pieces of uterus removed at operation were carefully noted at the time of removal; they were then fixed in Kaiserling's fluid. The colour was restored by treatment with 1% (w/v) sodium hydrosulphite in *N*/1 NaOH, after which the pieces were washed with water and transferred to glycerine. In this way the pieces of uterus removed at different stages could be compared directly as well as by comparison of the notes made at the operations. It was found that the colour of the uterus varied from the normal pinkish white to deep chocolate brown, and a scheme of assessment of the degree of pigmentation was drawn up as follows:

Colour	Rating
Normal (pinkish white)	0
Yellow	1
Dark yellow or orange	2
Orange brown	3
Red-brown or brown	4
Chocolate brown	5

Most of the animals received a small dose of synthetic *dl*-α-tocopherol and a larger dose of vitamin E concentrate, 1 ml. of the concentrate being equivalent to 8·75 mg. of the *dl*-α-tocopherol when measured by the amount required to maintain pregnancy in vitamin E-free rats. All the doses were given on consecutive days (omitting Sundays) in the period between operations. The concentrate was given undiluted in doses of 0·25 or 0·5 ml., and the *dl*-α-tocopherol as a solution in sesame oil, 10 mg./ml., the daily dose being 0·25 ml.

RESULTS

Tables 1 and 2 summarize the case histories of all the rats, the animals being arranged in groups according to age. The day of the first biopsy is called day 1, and all other stages of the experiment are given in days from this point.

The doses of vitamin E given are expressed in terms of *dl*-α-tocopherol and the amount received between operations is given in addition to the total dose. Where there were several matings and no pregnancy ensued, the number of matings is recorded, but not the actual days of mating. In these cases the final examination was delayed as it was not considered desirable to kill the animals until it became certain that they were infertile. Where there was more than one mating before implantation took place, the day of the last mating is recorded, followed by the number of matings in brackets.

Table 3 summarizes Tables 1 and 2. The animals are again grouped according to age, but the average rating of each of these age groups has been calculated, first at the beginning of the experiment and then after treatment with vitamin E. The results of treatment are divided into those in which treatment was followed by a successful pregnancy and those in which it was followed by resorption or birth of a dead litter. These groups are of necessity very small as they become subdivided according to response; for this reason, no statistical interpretation has been attempted. The figures give a clear indication that treatment with vitamin E alone has

Table 1. *The individual uterine condition, spacing of operations and time of mating, and the gestational history of vitamin E-deficient female rats before, during and after treatment with vitamin E*

Rat no.	Uterine condition Op. 1	Op. 2	Op. 3	Time of operation (day)	Time of mating (day)	Result of mating	Uterine condition post-mortem
1137	5	4	—	1 26	48	Resorption	3
1216	5	5	5	1 21 34	46	,,	1
1241	5	4	—	1 23	42	Litter 1 (still-born)	5
1091	3	4	—	1 25	47	Resorption	3
1541	3	—	—	1	—	—	0
1081	3	3	—	1 25	—	—	5
1079	4	3	—	1 25	48	Resorption	1
1482	4	—	—	1	20 (5 mates)	Litter 1	3
1542	4	—	—	1	20	Resorption	3
1448	4	4	—	1 25	51	Litter 3 (2 still-born)	0
1540	4	—	—	1	20	,, 5	0
1484	4	—	—	1	30	Resorption	3
1904	5	—	—	1	20 (2 mates)	Litter 7	1
1537	4	4	—	1 24	41	,, 2	0
1707	4	1	—	1 25	49	,, 7	0
1539	2	3	—	1 23	40	,, 3	1
1538	3	3	—	1 23	40	,, 4	2
1671	3	3	—	1 13	29 (2 mates)	Resorption	4
1668	2	2	3	1 19 34	66 (5 mates)	Litter 5	1
1708	0	0	—	1 14	30	Resorption	0
1917	5	—	—	1	19 (4 mates)	Litter 6	0
1903	4	—	—	1	57 (2 mates)	,, 5	0
1715	4	4	—	1 23	42	,, 6	1
1954	2	—	—	1	15	Resorption	3
1961	2	—	—	1	15 (3 mates)	Litter 4	0
1844	2	2	—	1 25	55	,, 7	1
1837	4	4	—	1 13	25	,, 7 (1 still-born)	0
1831	2	2	—	1 13	25 (2 mates)	,, 3	3
2081	0	—	—	1	29 (3 mates)	,, 6	0

Table 2. *The distribution and total doses of vitamin E given to vitamin E-deficient female rats (in terms of dl-α-tocopherol), and the period on a vitamin E-free diet before operation*

Rat no.	Months on diet	Vitamin E given (as mg. *dl*-α-tocopherol) As concentrate: Between Op. 1 and Op. 2	Between Op. 2 and Op. 3	Between last Op. and mating	As tocopherol on day of mating	Total dose: As concentrate	As *dl*-α-tocopherol
1137	10	35	—	40	5	75	5
1216	10	96	15*	0	5	96	20
1241	9	88	—	0	5	88	5
1091	8	35	—	40	5	75	5
1541	8	—	—	61	—	61	—
1081	8	70	—	0	—	70	—
1079	8	35	—	40	5	75	5
1482	8	—	—	66	5	66	5
1542	8	—	—	61	5	61	5
1448	8	35	—	40	5	75	5
1540	8	—	—	61	5	61	5
1484	8	—	—	61	5	61	5
1904	7	—	—	48	5	48	5
1537	7	—	—	83	5	83	5
1707	7	35	—	40	5	75	5
1539	7	83	—	0	5	83	5
1538	7	83	—	0	5	83	5
1671	7	35	—	57	5	92	5
1668	6	10*	96	0	5	96	15
1708	6	92	—	0	5	92	5
1917	6	—	—	48	5	48	5
1903	6	—	—	48	5	48	5
1715	6	88	—	0	5	88	5
1954	6	—	—	48	5	48	5
1961	6	—	—	105	5	105	5
1844	5	44	—	30	5	74	5
1837	5	92	—	0	5	92	5
1831	5	92	—	0	5	92	5
2081	4	—	—	83	5	83	5

* These doses were *dl*-α-tocopherol.

Table 3. *The average effect of treatment with vitamin E on the uterine colour rating of vitamin E-deficient rats treated after varying periods on a vitamin E-free diet*

Months on diet	Average rating	No. of rats	Average rating after treatment	No. of rats	Average rating after pregnancy	No. of rats	Average rating after resorption	No. of rats
4	0·0	1	—	—	0·0	1	—	—
5	2·7	3	2·7	3	1·3	3	—	—
6	2·7	7	2·2	3	0·4	5	1·5	2
7	3·5	6	2·8	5	0·8	5	4·0	1
8	3·7	9	3·5	4	1·0	3	2·5	4
9	5·0	1	4·0	1	—	—	5·0	1
10	5·0	2	4·5	2	—	—	2·0	2
Overall averages	3·3	29	3·1	18	0·75	17	2·6	10

very little effect on the uterine discoloration, the rating being reduced from 3·3 to 3·1, a reduction which obviously has no significance, whereas the same treatment followed by pregnancy and the birth of a living litter reduces the rating to 0·75, which must be considered significant, even on the small amount of available data. On the other hand, treatment with vitamin E followed by a gestation

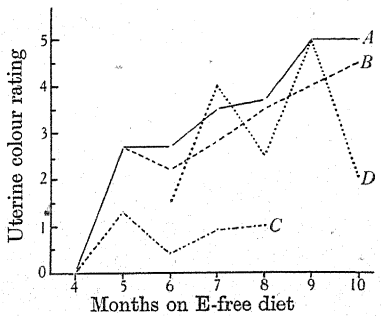

Fig. 1. Uterine colour rating before and after treatment with vitamin E. Curve *A*, before treatment. Curve *B*, after treatment. Curve *C*, after treatment and successful gestation. Curve *D*, after treatment and resorption, or unsuccessful gestation.

in which resorption occurred reduces the rating only to 2·6. In each treated group an improvement was obtained; this was only 0·2 after vitamin E treatment, 0·7 after vitamin E treatment and resorption, but 2·5 after vitamin E treatment and a successful gestation. A similar comparison can be obtained by considering the different age groups separately, but owing to the small number of animals, it would seem better to consider the averages of these groups. Fig. 1 clarifies this point by expressing the result graphically.

DISCUSSION

It appears clear from these results that the uterine discoloration produced by chronic vitamin E deficiency is a reversible condition, but that administration of vitamin E alone, even if given in massive doses, will have very little effect unless the animal is mated and gestation ensues. If the gestation is normal and a live litter results there is a marked, and in many cases, complete recovery from the pigmented condition. If a resorption gestation occurs the improvement is much less. It seems probable that pregnancy increases the circulation in the uterine muscle and is therefore the chief factor in the removal of the pigment.

Unfortunately, it is impossible to control the experiment by producing pregnancy and giving no vitamin E, as sterility is well established before the onset of pigmentation. It can only be shown that vitamin E alone is ineffective.

These results agree very well with those of Martin & Moore [1939], who found very similar uterine discolorations in their vitamin E-deficient rats. Unfortunately they did not examine the uteri before and after treatment, so that their results cannot be conclusive. These authors also say that after parturition the uterus of such rats remains abnormal, but since there was no previous examination, there is nothing to show that improvement has not taken place, and the foregoing experiments indicate that this was probably the case.

SUMMARY

1. Chronic vitamin E deficiency in the female rat produces uterine discoloration.

2. This discoloration is not curable by vitamin E administration alone, but yields to this treatment if accompanied by successful pregnancy.

3. It is suggested that the increased circulation to the uterine muscle during pregnancy is responsible for the removal of the pigment.

I wish to thank the Directors of the British Drug Houses Ltd., for permission to publish this work which was carried out while I was working in their laboratories.

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The Estimation of Creatine and of Diacetyl

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Voges & Proskauer [1898] observed that when strong alkali was added to broth cultures of certain species of bacteria there developed, after an interval of time, a pink colour. Subsequently the work of Harden [1906] and of Harden & Norris [1911] established that the chemical reaction responsible was one between diacetyl and creatine (or certain similar substances). Attempts to make the reaction quantitative [Walpole, 1911; Eggleton & Eggleton, 1928] were only partially successful. The reaction was further studied by Dulière [1929], Lang [1932] and Müller [1935], but it was the discovery by Barritt [1936], that α-naphthol greatly intensifies the colour, which made it possible for the first time to use this reaction as a means of estimating either creatine or diacetyl. The present paper presents some additional facts about the reaction and methods of estimation based on it.

EXPERIMENTAL

Preliminary experiments

Preliminary experiments confirmed Barritt's observations and also demonstrated that the reaction took place rapidly at room temp. Early in the research it was noticed that colour development invariably began at the surface of the liquid, suggesting that oxygen was involved in the reaction. This was proved by studying the reaction in the absence of O_2, and by measuring the amount of O_2 used. When the reagents were mixed under an atmosphere of O_2-free hydrogen, no colour formation occurred even after a period of 4 hr., whereas the control in air showed maximum colour development within 30 min. After 4 hr. exposure to O_2-free H_2, with no pigment production, air was admitted and immediately colour formation began and reached approximately the same intensity as the control.

The Warburg manometric apparatus was employed to measure the O_2 used during the reaction. Two ml. of the stock alkali (see later) or 2 ml. of 1% α-naphthol in the stock alkali, were placed in the main chamber of the manometer cup, and the creatine and the diacetyl solutions were placed in the side bulb. By this means the following points were established (for a temp. of 26°): (1) Creatine in alkaline solution does not absorb O_2. (2) α-Naphthol in alkaline solution consumes O_2 at a slow but steady rate over a period of many hours. (3) Diacetyl in alkaline solution shows a rapid initial O_2 uptake which falls off, sometimes completely, after 30 min. It amounts to about 1 mol. O_2/mol. diacetyl. (4) On mixing diacetyl with α-naphthol in alkali there is a consumption of O_2, the rate of which is approximately equal to the sum of the rates of O_2 consumption of diacetyl and α-naphthol separately. (5) The addition of diacetyl and creatine to the alkali causes a rapid consumption of O_2 for the first 30 min., which falls off to a low steady rate. The presence of excess of creatine increases by 2- or 3-fold the amount of O_2 consumed by diacetyl during this 30 min. In other words, O_2 is involved in the Voges-Proskauer reaction, though it is not possible to say how much, since under the conditions of these experiments some of the diacetyl must have oxidized without reacting with creatine. (6) When diacetyl and creatine are added to α-naphthol in alkaline solution, there is an initial rapid phase of O_2 consumption lasting about 30 min., followed by a slow steady O_2 uptake lasting over a period of hours. The amount of O_2 used by this mixture is greater than when creatine is omitted, indicating that the modified Voges-Proskauer reaction also involves an oxidation by molecular O_2. The results of a typical experiment are expressed graphically in Fig. 1. In this experiment, the amount of

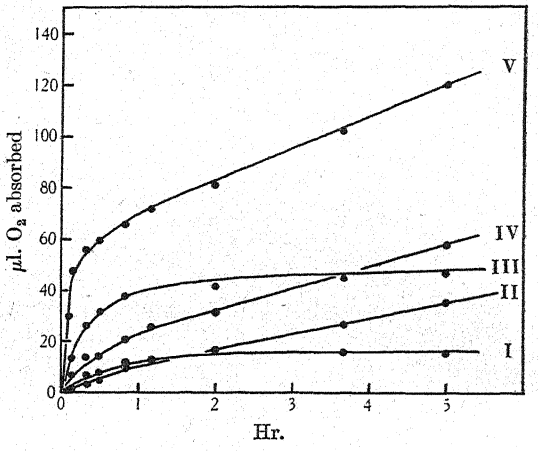

Fig. 1. O_2 uptake at 26° (measured in Warburg manometers) of the following mixtures dissolved in 2 ml. of stock alkali.

I.	α-naphthol	0 mg.,	diacetyl	0·2 mg.,	creatine	0	mg.
II.	,,	10 ,,	,,	0·0 ,,	,,	0	,,
III.	,,	0 ,,	,,	0·2 ,,	,,	7·5	,,
IV.	,,	10 ,,	,,	0·2 ,,	,,	0	,,
V.	,,	10 ,,	,,	0·2 ,,	,,	7·5	,,

diacetyl (0·2 mg.) was the limiting factor, creatine and α-naphthol being present in excess, 7·5 and 10 mg. respectively. (7) The rate of O_2 consumption is approximately equal to the rate of colour development. The rate of O_2 consumption was obtained as the difference between the rate of O_2 consumption of a mixture of diacetyl, creatine and

α-naphthol and the rate of O_2 consumption of a similar mixture of diacetyl and α-naphthol. Colour development was measured by means of the Zeiss Pulfrich photometer. Within the limits of experimental error, which in this case are large, owing to the speed of the reaction and the presence of side reactions, colour development and O_2 consumption ran parallel.

These experiments show that the Voges-Proskauer reaction and the Barritt reaction both involve an oxidation by molecular O_2, and in consequence special attention must be paid to this fact when using the reaction for the estimation of either creatine or diacetyl.

Nature of the pigment formed in the Voges-Proskauer and Barritt reactions

The isolation of the pigment formed in the modified reaction has been attempted, but so far it has proved impossible to obtain it in the crystalline state. The impure product has some interesting properties. In the solid state it has been stable for 4 years. In alkaline solutions of pH 10 it is coloured red; at pH 8 it is purple, and at pH 6 it is brown; it is least soluble at pH 4. These facts suggest that it is a compound with three ionizing groups and an isoelectric point at pH 4. In concentrated HCl or H_2SO_4 the pigment is quite stable and coloured purple. In solution at pH 12, 40 mg. impart the same intensity of colour as 7 mg. of creatine in excess of diacetyl and α-naphthol, or 5 mg. of diacetyl in excess of creatine and α-naphthol.

The pigmented solution produced in the presence of α-naphthol was examined spectroscopically (our thanks are due to Dr L. E. Bayliss for valuable advice and help) and the absorption spectrum was found to have a single peak centred on wave-length 5250 A., the extinction coefficient (E) having half the peak value at 4650 and 5660 A.

The Zeiss S 50 and S 53 filters proved to be suitable for the photometric measurement of the pigment. The question of the optimum conditions for the development of maximum colour from small amounts of creatine and small amounts of diacetyl was then investigated.

Investigation of reaction conditions

Alkali. A solution of NaOH was used both in the original Voges-Proskauer test and in the Barritt modification. Investigation of the degree of alkalinity necessary for maximal colour development in the Barritt reaction showed that (*a*) no colour developed when the solution was made alkaline with $NaHCO_3$, (*b*) pigment formation occurred when either Na_2CO_3 or NaOH was used, and (*c*) maximum colour development occurred when a mixture of NaOH and Na_2CO_3 was used. The concentration of these compounds was not critical. A stock alkali solution was made up containing 30 g. NaOH and 80 g. Na_2CO_3 (equimolar amounts) in distilled H_2O and made up to 500 ml. Two ml. of this solution were used for the estimation.

α-Naphthol. Barritt used a solution of α-naphthol in ethanol, but as this rapidly becomes pigmented on standing, a solution in the stock alkali was used instead. The amounts of α-naphthol required for maximal colour development proved to be surprisingly large, about 500 mol./mol. of creatine, and at first the activity of the α-naphthol was thought to be due to the presence of some impurity. However, after thrice distilling in steam from an acid solution, the α-naphthol was neither more nor less active than before. Steam distillation yielded a perfectly white, crystalline product, which, if kept in a dark bottle, did not become discoloured. All subsequent samples of α-naphthol were purified in this manner. The solution of α-naphthol in alkali does not keep more than 2 or 3 hr. and in consequence it was always made up just before it was required. A 1 % solution was used.

Diacetyl. A stock solution is prepared from dimethyl glyoxime (British Drug Houses Ltd., 'Analytical Reagent') by heating 1·6 g. with 200 ml. of 5 N H_2SO_4 in an all-glass distilling apparatus and collecting the first 50 ml. of distillate; this is made up to 100 ml. with H_2O. The amount of dimethylglyoxime taken yields about 1 g. diacetyl (as assayed by the van Niel [1927] method). This yield (85 % of theoretical) is fairly constant, and the solution is stable for at least a month if kept in the ice-box.

Excess diacetyl causes production of a yellow pigment which tends to obscure the colour produced by the creatine; a deficiency causes subnormal colour production. For amounts of creatine up to 60 μg. in a final volume of 10 ml., 1 ml. of 1 : 20 dilution of the stock 1 % solution was found to be optimal.

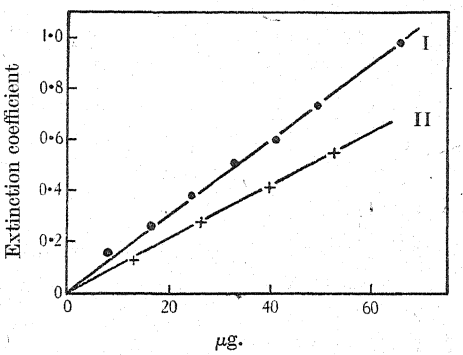

Fig. 2. Colour intensity (measured as extinction coefficient, S 53 filter, 10 mm. cuvettes) produced by different amounts of creatine (II) and diacetyl (I) when treated in the manner described in the text.

Creatine. The sample used (British Drug Houses Ltd.) was recrystallized from distilled H_2O. With 0–50 μg. of creatine the colour was proportional to the amount of creatine present. Similarly, it was shown that when creatine was in excess the colour was proportional to the amount of diacetyl present. The results (Fig. 2) show that there is a linear relationship between intensity of colour and amount of diacetyl, and that the chromogenic powers/μ.mol. of diacetyl and creatine are about equal. This may indicate that the pigment contains diacetyl and creatine in equimolar proportions.

METHODS OF ESTIMATION

(*a*) *Creatine*

Reagents. (1) Stock alkali. (2) Stock 1 % diacetyl diluted 1 : 20 before use. (3) α-Naphthol purified by steam distillation. A 1 % solution in the stock alkali is made up just before use. (4) Saturated solution of creatine.

Procedure. To a neutral solution of creatine (containing not more than 60 μg.) in a standard

10 ml. flask are added 2 ml. of the 1% α-naphthol in alkali followed by 1 ml. of the diluted diacetyl. The solution is briskly shaken and made up to the mark with distilled H_2O and left for 30 min. The colour may be measured with the Zeiss Pulfrich photometer (S 53 filter and a reagent blank in the control cuvette), or by direct colorimetry with standard creatine solutions prepared in the same way. There is no interfering colour from the reagents alone and the intensity of the colour is stable for at least 2 hr. The extinction coefficient for creatine under these conditions is 0·0105/μg. (10 mm. cuvette, and S 53 filter).

Many guanido compounds react under these conditions [cf. Barritt, 1936]. Of those that we examined quantitatively, arginine, guanidine and glycocyamine produce about one-ninth of the colour given by an equivalent amount of creatine. Creatinine and freshly prepared creatine phosphate do not react under these conditions.

(*b*) *Diacetyl*

Reagents. As for the estimation of creatine (see p. 527).

Procedure. To a solution of diacetyl (containing not more than 100 μg.), in a standard 15 ml. flask, are added 4 ml. of a solution made up from 1 part saturated aqueous creatine and 3 parts 1% α-naphthol in stock alkali. The flask is briskly shaken, made up to the mark with distilled H_2O and allowed to stand for 30 min. It is then estimated in the photometer with a reagent blank in the control cuvette, or by direct vision colorimetry. Of the two the latter method is to be preferred owing to the presence of a slight variable blank error which it has proved impossible to eradicate.

Diacetyl does not appear to be formed by cells to any large extent; its reduction product, acetyl methyl carbinol, is far more common. This substance is readily oxidized to diacetyl by refluxing with ferric chloride [van Niel, 1927] and the diacetyl so formed may be removed by distillation, e.g. in the micro-Kjeldahl apparatus provided by Quickfit and Quartz Ltd. The following experiment illustrates the speed with which this occurs. 1 ml. of diacetyl solution containing 163 μg. diacetyl was pipetted into each of four distillation flasks and distilled in turn for varying periods of time. The distillate and washings were collected in calibrated measuring cylinders, the volume noted and 5 ml. taken in each case for analysis (Table 1).

Table 1. *Recovery of diacetyl by distillation*

Exp. no.	1	2	3	4
Diacetyl added (μg.)	163	163	163	163
Time of distillation (min.)	5	10	15	20
Vol. of distillate washings (ml.)	8·8	11·4	15·4	22
Diacetyl recovered (μg.)	164	162	154	159

In Exp. 1, 5 ml. of distillate were collected, the remainder coming from washings. It appears therefore that by collecting 10 ml. of distillate a quantitative recovery is assured.

Sources of interference

(1) The use of $CCl_3.COOH$ as a protein precipitant can cause some interference, since it reacts with alkaline α-naphthol solutions, producing an intense blue pigment which entirely masks the colour given by creatine. This reaction occurs only in ultra-violet light. If the colour development is carried out in the dark room or in the light of a tungsten filament lamp, $CCl_3.COOH$ does not interfere.

(2) Urine, and $CCl_3.COOH$ extracts of liver, contain substances which inhibit the reaction between diacetyl, creatine and α-naphthol. (The reaction between creatine and diacetyl is similarly inhibited, and Walpole believed that NH_3 was responsible for the inhibition in the case of urine.) For example, the colour production from 30 μg. creatine in excess diacetyl was reduced to 74% by the presence of 1 ml. of a normal urine, the effect being proportional to the amount of urine added. Similarly the colour intensity due to diacetyl in the presence of excess creatine was reduced to 68% of normal by 1 ml. of the same urine. This marked inhibitory action was not shown by urea, NH_3, uric acid or creatinine when added in amounts likely to be present in 2 ml. of urine. Glycine, however, proved to be an inhibitor. The colour intensity with 30 μg. of creatine was reduced by one-half in the presence of 4 mg. of glycine. Alanine and glutamic acid did not show this property, but β-alanine and β-alanyl histidine (carnosine) were inhibitory, being about 60% as active, on a molar basis, as glycine.

These substances also inhibited the colour production by diacetyl in the presence of excess creatine, indicating that the mechanism of the interference is not a destruction of either diacetyl or creatine, but a reaction with some compound formed intermediately in the Barritt reaction.

Discussion of methods

It appears from the above experiments that omega amino-acids may be responsible for the inhibitory action of liver extracts and urine. No ornithine or lysine was available so it was impossible to test the hypothesis that all omega amino-acids inhibit the reaction, nor was the effect of primary amines tried, though it may well be that the inhibition is due to the terminal amino group $—CH_2.NH_2$. In the light of these experiments, the claim of Lang [1932] to be able to estimate arginine in protein digests by a diacetyl method seems to require confirmation. Indeed, we observed no colour in a neutralized acid hydrolyzate of gelatine which had been treated with excess diacetyl and α-naphthol in alkaline solution, despite the presence of large quantities of arginine.

The use of the method for the estimation of arginine or creatine is therefore limited by these inhibitors. However, most animal tissues, with the

exception of liver, appear to contain insignificant quantities of interfering substances. In the case of skeletal muscle with its relatively high content of β-alanine in the form of carnosine and anserine, the situation is mitigated by the fact that the creatine content is also very high. Thus, only 0·1 ml. of a 1 : 10 CCl_3.COOH extract of skeletal muscle is required for the estimation of free creatine, and in this small volume the concentration of β-alanine-containing dipeptides is negligible. None the less, if the method is used in connection with muscle creatine, the possibility of interference from these sources should be borne in mind. It is possible that a preliminary separation of the creatine by selective adsorption would overcome the difficulties. In the estimation of diacetyl the preliminary distillation removes these sources of interference.

RESULTS

Except in the case of liver, no noticeable abnormalities have been encountered with animal tissues. Table 2 gives figures obtained with neutralized 1 : 10 CCl_3.COOH extracts of certain tissues of the rat. The animals were killed by stunning.

Table 2. *Creatine content of animal tissues*

Tissue	Present method—rat (mg./100 g.*)	Picrate method [Hunter, 1928] (mg./100 g.*)
Skeletal muscle	524	470
Cardiac muscle (ventricle)	192	168–223 (rabbit)
Smooth muscle (small intestine)	30·4	27·1 (rabbit)
Whole brain	132	114 (rabbit)
Lung	8·1	No figures
Spleen	6·5	19·1
Kidney	19·9	15·8–29 (dog)
Testis	267	212 (sheep)
Liver	0	23·3 (rabbit)

* Wet weight.

A portion of the tissue was removed, freed from blood by washing with distilled H_2O, dried on filter paper, weighed, and ground with 10 times its weight of 4% CCl_3.COOH and a little sand. The insoluble material was filtered off and portions of the clear filtrate neutralized before development of the colour. It is assumed that the pigment formed was entirely derived from creatine. For comparison are given figures taken from Hunter [1928]; where no figures for the creatine content of rat tissues were available, those for corresponding tissues of other species are quoted. Hunter's results were all obtained by converting the extracted creatine into creatinine and determining the creatinine by the Jaffé reaction.

SUMMARY

1. Barritt's observation, that the Voges-Proskauer colour test for diacetyl is greatly increased in sensitivity in the presence of α-naphthol, is confirmed.

2. Barritt's reaction can also be used as a sensitive test for creatine.

3. Both the Voges-Proskauer and the Barritt reactions involve an oxidation by molecular oxygen.

4. Certain amino-acids interfere with the colour production in the Barritt reaction when the latter is used to estimate either creatine or diacetyl.

5. A similar interference is noted when urine and extracts of liver are present in the reaction mixture.

6. Arginine, guanidine, and glycocyamine react in a similar manner to creatine, but produce less colour. Creatinine and creatinephosphoric acid produce no colour.

7. Methods are described for the estimation of creatine and of diacetyl, in quantities up to 50 μg.

8. A reaction between α-naphthol and trichloroacetic acid is described, which occurs only under ultra-violet excitation.

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Vitamin Methods

7. A NEW MODIFICATION OF THE *p*-AMINOACETOPHENONE METHOD FOR ESTIMATING NICOTINIC ACID IN URINE

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(*Received* 1 *September* 1943)

Harris & Raymond [1939] developed a method for estimating nicotinic acid in urine with the object of assessing the nutritional status in this vitamin, along the same lines as those employed in the saturation tests previously devised for vitamin C [Harris & Ray, 1935] and vitamin B_1 [Harris & Leong, 1936]. The method was based on the cyanogen bromide reaction of König [1904], a novel feature being the reagent used as the aromatic amine, namely, *p*-aminoacetophenone. Although other aromatic amines have also been used for determining nicotinic acid, e.g. aniline and *p*-aminophenol, several investigators have concluded that Harris & Raymond's reagent is the most advantageous [Naganna, Giri & Venkatesam, 1941; Stotz, 1941; Arnold, Schreiffler & Lipsius, 1941].

With urines and highly coloured extracts the colour reaction is always less easy to perform than with colourless material. To overcome this difficulty, several alternative procedures have been suggested, but some of these are too tedious, while others run the risk of loss of nicotinic acid. For this reason we have undertaken an extensive study with special reference to the following: the removal of interfering coloured and non-coloured substances; the problem of the 'blank' correction; the conditions of the preliminary conversion of related substances to nicotinic acid. The optimal conditions for the reaction of CNBr with nicotinic acid and the subsequent development of colour [Harris & Raymond, 1939; Kodicek, 1940 *a*, *b*] have been reinvestigated with regard to the complex composition of urinary extracts.

As a result, an improved modification of the *p*-aminoacetophenone method has been devised, which has the advantage of being simple and reliable. The method, which is based on the original procedure of Harris & Raymond [1939], has been used in this laboratory for over two years with good results.

GENERAL CONSIDERATIONS

(1) *Removal of interfering colour*

There are, in general, three methods of estimating a substance in a coloured solution: (*a*) by removing the substance from the solution, thus freeing it from the interfering colour; (*b*) by eliminating the colour from the solution without losing the compound to be determined; and (*c*) by estimating it in the coloured solution and allowing for the colour by a blank correction.

Separation of nicotinic acid from urine by removing it on adsorbents or by extracting it with an immiscible solvent is not very practicable owing to the difficulty of effecting a complete separation, and furthermore, some pigment is removed with the vitamin. A further disadvantage is the difficulty of eluting the vitamin quantitatively after adsorption. Perlzweig, Levy & Sarret [1940] claimed to have obtained good results by using Lloyd's reagent after preliminary oxidation. Dann & Handler [1941], using Perlzweig's decolorization procedure, confirmed this finding. The decolorization procedure, however, is more tedious than the oxidation method described in this paper and is much dependent upon various physical properties of the adsorbing agent. The adsorption of nicotinic acid by medicinal charcoal, as suggested by Giri & Naganna [1941], failed to give, in our hands, reproducible results. Swaminathan [1939] used charcoal in a different manner—to remove pigments from the urine, leaving nicotinic acid in the solution. He thereby eliminated the use of a blank correction except in cases in which the test solutions were still coloured. The adsorption was carried out in an alkaline medium, and it was claimed that no nicotinic acid was lost by this treatment. Melnick & Field [1940 *a*, *b*], like Swaminathan, used charcoal to remove interfering pigments, but performed the adsorption in acid alcoholic solutions. They admitted that their method was liable to give results with urine which are too high.

The method to be described combines principles (*b*) and (*c*) mentioned above, without recourse to adsorption. It consists of a preliminary washing of the hydrolyzed urinary solution with isobutanol, which removes part of the interfering coloured substances, and a subsequent oxidation of the remaining colour with potassium permanganate. The treatment usually produces a solution which is almost colourless. Any residual coloured or colourless interfering substances are allowed for by a special blank correction.

(2) *The blank correction*

Several ways of allowing for the blank correction have been suggested by various workers, but none of them seems to be entirely satisfactory. Bandier [1939*b*] used as a blank a sample of the urine treated as the 'unknown' but without the addition of the amine. The inadequacy of this blank, at least for *p*-aminoacetophenone, will be shown later when the different types of blank are compared. Harris & Raymond [1939] omitted CNBr from their blank, which had otherwise been treated exactly like the 'unknown'. This has been

criticized by Melnick & Field [1940*b*] on the ground that the amine reacts with the interfering constituents of urine to form a colour which is not produced in the presence of CNBr. We could not observe, however, any bleaching effect of CNBr under our experimental conditions, nor could we find in our new 'acid blank' any unspecific side reaction which would not be present also in the 'unknown' [cf. Melnick, Oser & Siegel, 1941]. Dann & Handler [1941] state that the cyanogen bromide procedure worked well only in a colourless solution. They adopt the procedure of Perlzweig *et al.* [1940] to produce an almost colourless extract, and introduce a blank correction which consists of the residual colour of the extract and the colour of the reagents measured separately. Although we agree with Dann & Handler that a colourless extract is to be preferred to a coloured one, we feel that their blank correction may show, with certain extracts, too low a value. Their blank is based on the assumption that a colourless extract contains no interfering substances which may react with the reagents to give a colour. We find that occasionally an apparently colourless solution may still react with the reagents to produce a slight colour. In the procedure to be described we have included all the reagents and the test solution in one blank carried out in such a way that the nicotinic acid in the test solution does not react.

(3) *Preliminary conversion to nicotinic acid of related substances*

Another point which has been the subject of much controversy among different workers is the question whether the conversion to nicotinic acid of related substances should be carried out in acid or in alkaline solution. Swaminathan digested the urine in 2 *N* NaOH for a period of 3 hr. Under such conditions, a considerable portion of trigonelline may be changed to nicotinic acid, which may account for his high values for nicotinic acid output. Melnick & Field, on the other hand, subjected the urine to acid hydrolysis. As pointed out by Perlzweig *et al.* [1940], this has the danger of preventing the complete conversion of nicotinuric acid to nicotinic acid. In the method of Harris & Raymond the digestion was carried out in *N* NaOH for 30 min. It will be shown later that this short digestion with the relatively dilute alkali is preferable, as it does not cause any appreciable conversion of trigonelline to nicotinic acid even in the presence of large quantities of the former compound. Moreover, it is much more convenient, and yields a lighter coloured solution than the method with acid digestion.

(4) *Optimal conditions for the König reaction and subsequent development of colour*

The various factors which influence the König reaction have been extensively investigated by Harris & Raymond [1939] and Kodicek [1940*a*]. There were, however, a few more points which needed further study, especially the conditions for the reaction of CNBr with nicotinic acid. All these factors have been investigated in the present work, and are discussed below in §§ IV and V.

EXPERIMENTAL

(I) *Comparison of preliminary acid and alkaline digestion*

Three 40 ml. portions of a sample of urine were digested with 20 ml. of 12 *N* HCl on a boiling water-bath for 5 hr., neutralized, and decolorized as described in § II(*a*) below. Further batches of eight 50 ml. portions of the same urine, four with added trigonelline and four without, were digested with *N* NaOH for 45 min. on the water-bath, neutralized and decolorized as before. They were then analyzed for nicotinic acid. It will be seen from Table 1 that the same values were obtained whether the digestion was carried out in acid or alkali, and, in the latter case, with or without the addition of trigonelline. The slight variations in the individual values are due to the experimental error, since, with the low values of extinction, the personal error in reading is from 5 to 10%.

Table 1. *Comparison of results with preliminary alkali and acid digestions*

Estimations of nicotinic acid in the presence and absence of trigonelline

	Digestion in alkaline solution				Digestion in acid solution.
	Extinction with				
Material	Acid blank	Urine	Urine +10 μg. of nicotinic acid	Nicotinic acid found in urine (μg./ml.)	Nicotinic acid found in urine (μg./ml.)
Urine alone: Test 1	0·049	0·135	0·352	0·79	0·74
Test 2	0·052	0·141	0·355	0·83	0·73
Test 3	0·049	0·125	0·321	0·78	0·77
Test 4	0·049	0·146	0·350	0·95	—
Average	0·050	0·137	0·341	0·84	0·75
Urine, same specimen +0·5 mg. of trigonelline/10 ml.	0·048	0·142	0·346	0·92	—
Urine, same specimen +0·5 mg. of trigonelline/10 ml.	0·054	0·132	0·336	0·77	—
Average	0·051	0·137	0·341	0·84	—
Urine, same specimen +1·0 mg. of trigonelline/10 ml.	0·053	0·157	0·381	0·93	—
Urine, same specimen +1·0 mg. of trigonelline/10 ml.	0·055	0·146	0·360	0·85	—
Average	0·054	0·152	0·371	0·89	—

(II) *Decolorization of urinary digests*

After the preliminary digestion, which serves to convert related substances to nicotinic acid, the urine usually becomes quite dark in colour. It is necessary to remove the colour; otherwise the blank correction will be too high and the accuracy of the method much decreased. Since digestion with alkali always yields a lighter coloured solution than digestion with acid, and as shown above, trigonelline does not interfere, it was decided to use the former method of digestion and to carry out the decolorization of this solution. Two methods of decolorization were tried:

(*a*) *Charcoal adsorption.* The procedure used for decolorization with charcoal as recommended by Swaminathan [1938] was used with some modifications. The method is as follows:

After the preliminary digestion with alkali (unlike Swaminathan, we digested with *N* NaOH, i.e. 5 ml. of 40% (w/v) NaOH added to 50 ml. of urine, and the time of digestion restricted to 45 min.), the urine was neutralized with conc. HCl, and then adjusted to a less strongly alkaline reaction by the addition of 0·5 ml. of 40% NaOH. The precipitate was centrifuged off. The centrifugate was made up to 50 ml., and boiled with 1 g. of norite charcoal. The solution was filtered hot with suction and the charcoal on the filter paper boiled in about 30 ml. of dilute alkali (0·3 ml. of 40% NaOH to 100 ml. of water) for 1–2 min., filtered again and the residue washed with 20 ml. of the hot dilute alkali. The combined filtrates were brought to pH 6·5–6·8 with HCl and made up to 100 ml. The solution was then analyzed for nicotinic acid according to the procedure described below.

This procedure for decolorization gave good recoveries (Table 2). Larger amounts of charcoal (2 g. as used by Swaminathan), however, caused 20–40% loss of added nicotinic acid. The slight yellow colour that remained after adsorption was allowed for by a blank correction. However, owing to the difficulty of obtaining charcoal of uniform quality, this procedure was given up in favour of the second method to be described, which not only gives a more colourless extract but also has other advantages and gives more reliable results.

Table 2. *Recovery of added nicotinic acid after decolorization of urine with charcoal*

Exp. no.	Nicotinic acid in original urine (μg./ml.)	Nicotinic acid added (μg./ml.)	Additional nicotinic acid found (μg./ml.)	Recovery (%)
1	5·1	10·0	9·8	98
2	1·7	10·0	11·2	112
3	4·0	10·0	10·3	103
4	1·24	1·00	1·16	116
5	1·05	2·00	2·15	108
6	0·58	2·00	2·32	116
7	0·48	2·00	1·98	99
8	1·0	0·93	0·74	80
			Average	104

Standard deviation of single determination ±12.

(*b*) *Washing with isobutanol and oxidation with* $KMnO_4$. Urinary pigments may be effectively removed from solution by extracting them with immiscible solvents, such as phenol or benzylalcohol, but unfortunately a great proportion of nicotinic acid is removed as well. Isobutanol extracts, albeit incompletely, a considerable part of the urinary pigments, while it leaves practically all the nicotinic acid behind. The distribution coefficient of nicotinic acid between water-saturated isobutanol and isobutanol-saturated water below pH 4·5 is about 1 : 19. By using 1 vol. of isobutanol to every 2 vol. of urinary digest we have found it possible to remove a large part of the pigments without losing any significant amount of the nicotinic acid (Table 3), but for very accurate work, a correction factor could be introduced to account for the amount extracted by the isobutanol. The residual colour is then removed by oxidation with $KMnO_4$. The oxidation is carried out in acid solution at about 50°. A 4% solution of $KMnO_4$ is added drop by drop with continuous stirring until the colour of the permanganate stays for about 30 sec. The colour is allowed to disappear completely. This usually takes 5–10 min.* The solution is then neutralized carefully to pH 6·5–

Table 3. *Recovery of nicotinic acid after a solution in M/45 phosphate buffer* (pH 4·5) *had been washed with isobutanol*

	Extinction	Amount found (μg.)	Recovery (%)
(*a*) 10 μg. of nicotinic acid present:			
After washing with isobutanol	0·200	10·5	105
Control without treatment	0·251	10·4	104
(*b*) 25 μg. of nicotinic acid present:			
After washing with isobutanol	0·786	23·8	95
After washing with isobutanol	0·809	24·2	97
Control without treatment	0·852	25·7	103

Table 4. *Recovery of added nicotinic acid with the new method*

	Nicotinic acid			
Exp. no.	In original urine (μg./ml.)	Added (μg./ml.)	Additional found (μg./ml.)	Recovery (%)
1	1·5	2·5	2·34	94
2	0·82	2·5	2·7	108
3	0·89	2·5	2·3	92
4	0·87	2·5	2·48	99
5	0·93	2·5	2·44	98
6	0·61	2·5	2·59	104
7	0·82	2·5	2·3	92
8	0·96	2·5	2·59	104
9	1·15	2·5	2·55	102
10	0·63	2·5	2·39	96
11	0·83	2·5	2·32	93
12	1·17	2·0	2·09	105
13	0·26	2·0	1·85	93
			Average	98·5

Standard deviation of single determination ±5·6.

* If the liquid is not sufficiently reducing, as with a solution of the pure vitamin, the excess of permanganate has to be reduced by the addition of a few drops of 0·5% H_2O_2. The excess of H_2O_2 must be removed (e.g. by catalase), as otherwise it may interfere with the subsequent development of colour.

6·8. Any manganous hydroxide separating out is centrifuged off. The solution at this stage should be practically colourless or of light straw colour and is ready for treatment with CNBr. Recovery of added nicotinic acid in solutions so treated is complete (Table 4). The advantage of this oxidation method is not limited to the production of an almost colourless extract. It gives also a solution free from reducing substances, such as ascorbic acid or sulphites, which when present in large amounts may prevent the proper development of colour. Other reducing substances so far tried (sodium thiosulphate, uric acid) had the same effect. The presence of such substances decreases the sensitivity of the method, and the advantage of the oxidation procedure is that it eliminates this interference. Hence the new modification seems to be a further improvement in comparison with other methods.

(III) *Comparison of different blanks*

Three different blanks were tried, (*a*) 'CNBr blank', i.e. a sample of urine treated in the same manner as the 'unknown' but without the amine; (*b*) 'amine blank', i.e. without CNBr; and (*c*) 'acid blank', see below.

Theoretically no blank can be considered perfect which omits any of the reactants. The 'CNBr blank' advocated by Bandier and described above, errs probably on the low side. The 'amine blank' of Harris & Raymond gives a higher correction, but the fact that CNBr is omitted might be criticized. It was found that when nicotinic acid was treated with CNBr in an acid solution, no reaction took place. This observation makes it possible to include all the reactants in the blank by simply adding acid to the test solution in the reversed order, namely, *before* treatment with CNBr. This blank is referred to in future as the 'acid blank'. It will be seen from Table 5 that the value of this blank is

Table 5. *Nicotinic acid estimated in urines with use of different blanks and charcoal decolorization*

	Nicotinic acid found (mg./day) with				
Sample	Urine blank*	CNBr blank	Amine blank	Amine blank plus CNBr blank	Acid blank
1	4·40	3·88	2·88	2·37	2·24
2	3·38	2·83	2·65	2·06	2·21
3	3·01	2·62	1·98	1·51	1·56
4	6·04	6·04	4·78	4·78	4·68
5	4·90	4·20	2·66	1·95	1·85
6	2·69	2·27	1·87	1·46	1·37
7	2·92	2·50	1·54	1·13	1·12
8	3·58	3·34	1·98	1·74	1·77
9	3·30	2·85	2·02	1·55	1·55
10	1·80	1·54	0·99	0·73	0·71

* Urine treated exactly in the usual manner, but without the addition of CNBr and amine reagent.

approximately equal to the 'CNBr blank' and the 'amine blank'. When the washing with isobutanol and the oxidation by $KMnO_4$ were used, the 'acid blank' showed even lower values, corresponding approximately to the sum of the original colour of the CNBr and amine. It is interesting to note that the values of normal human excretion of nicotinic acid obtained by means of this new blank tally well with those of Perlzweig *et al.* [1940] and of Snell & Wright [1941] determined by a different technique.

(IV) *Optimum conditions for the reaction of* CNBr *with nicotinic acid*

In their original method, Harris & Raymond used a temperature of 80° and a duration of 10 min. for the reaction. Kodicek [1940*b*] allowed 5 min. for the reaction to take place. The effect of both temperature and time was reinvestigated by varying these two factors.

(*a*) *Temperature.* A solution of pure nicotinic acid containing 2 μg./ml. was used. The CNBr was added and the reaction mixture placed for 5 min. on a water-bath maintained at constant temperature. The results are given in Table 6. It will be seen that the optimum temperature for maximal colour production is between 50 and 60°.

Table 6. *Effect of temperature on reaction of* CNBr *with nicotinic acid*

Temp. (° C)	Extinction
20	0·148
40	0·305
50	0·388
60	0·418
70	0·329
80	0·236

(*b*) *Time.* The same solution of nicotinic acid was used, and the temperature was kept at 56–58°. The reaction mixture was placed in the water-bath for varying lengths of time. As illustrated in Table 7, the highest extinction was obtained within 2–5 min. It was, therefore, decided to use 4 min. for the reaction in our procedure.

Table 7. *Effect of time on reaction of* CNBr *with nicotinic acid*

Time (min.)	Extinction	
	Exp. 1	Exp. 2
2·0	—	0·408
2·5	0·408	—
3·5	—	0·408
5·0	0·410	0·410
10·0	0·335	—
20·0	0·267	—

(V) *Optimum conditions for the development of colour*

(*a*) *Amount of amine.* It was felt that the amount of amine used previously by Harris & Raymond [1939] and Kodicek [1940 *a*, *b*] might not be the optimum amount for

Table 8. *Effect of variation in the amount of amine used*

10% ethanolic *p*-aminoacetophenone added (ml.)	Extinction for solution of nicotinic acid containing (μg.)	
	10	20
0·2	0·218	0·469
0·3	0·246	0·503
0·6	0·292	0·585
0·9	0·314	0·635
1·2	0·311	0·633
1·5	0·320	0·648

the reaction, as has already been pointed out [Kodicek, 1940*a*]. The fact that the urinary extract was different in composition from other extracts used by one of us (E.K.) made it necessary to reinvestigate this point. It is clear from Table 8 that a nearly optimum development of colour is reached with the addition of about 1·0 ml. of a 10% solution of *p*-aminoacetophenone in ethanol.

(*b*) *Effect of acid upon the final development of colour.* The intensity of colour produced by the reaction between the amine and the CNBr-treated nicotinic acid depends on the acidity of the solution. The optimal acidity corresponds to a pH of about 1·5, as illustrated by Table 9. It will be seen that the intensity of colour varies only slightly with changes of acidity below a pH value of 2. It was found that 0·6 ml. of a 10% HCl was sufficient to bring the pH of the urines so far examined to a value between 1 and 2 under the conditions of our procedure.

Table 9. *Effect of amount of acid*

10% HCl (ml.)	Extinction for 10 μg. nicotinic acid in *M*/45 phosphate buffer of pH 6·6	pH (quinhydrone electrode)
0·1	0·177	2·51
0·2	0·232	2·14
0·3	0·252	1·87
0·4	0·257	1·68
0·6	0·275	1·45
1·0	0·243	1·13

(*c*) *Stability of the colour produced.* It will be seen from Table 10 that the colour is fairly stable and there is very little fading after standing in the dark for periods up to 30 min. This agrees with the findings of Harris & Raymond [1939].

Table 10. *Fading of developed colour*

Time (min.)	Extinction for solution of nicotinic acid containing (μg.) 12·5	25	50
5	0·255	0·500	1·010
10	0·263	0·519	1·030
15	0·264	0·517	1·017
20	0·259	0·514	1·022
25	0·253	0·514	1·010
30	0·253	0·518	1·008
60	0·239	0·480	0·948

METHOD ADOPTED

Reagents

CNBr *solution.* Prepared fresh daily by titrating a saturated solution of ice-cold bromine water with 10% ice-cold NaCN solution until the colour of bromine disappears. (NaCN was found to be better than KCN.)

Alcoholic amine reagent. 10 g. of recrystallized *p*-aminoacetophenone were dissolved in 96% ethanol and made up to 100 ml. The solution should be prepared fresh every week as older solutions tend to deepen in colour. (The *p*-aminoacetophenone was recrystallized from a warm saturated solution in 96% ethanol by adding about 3 vol. of distilled water. After several hours at 0° the precipitate was filtered off. The process of crystallization may be repeated a second time if necessary. The purified product was dried *in vacuo* over $CaCl_2$. It should be of a light creamy colour.)

HCl *solution* 10% w/w.

Concentrated HCl *solution* 36% w/w.

NaOH *solution* 40% w/v.

$KMnO_4$ *solution* 4% w/v.

Isobutanol. Redistilled and saturated with water.

Standard solution of nicotinic acid. 20 μg./ml. in 25% ethanol. Prepared weekly from a stronger solution containing 1 mg./ml. in 25% ethanol.

Procedure

(1) *Preliminary digestion.* If the total amount of urine excreted each day is less than 1500 ml., dilute the urine to this volume. Take 20 ml. of urine, add 2 ml. of 40% NaOH and heat on a boiling water-bath for 45 min. Cool, adjust pH to 2–3 with conc. HCl, with thymol blue as external indicator. Make up the volume to 25 ml. with distilled water. Pour the treated urine into a 50 ml. separating funnel; add isobutanol corresponding to one-half the final volume of digested urine and shake for 2 min. Allow to separate, then run out the aqueous layer. Take 20 ml. of this water layer and evaporate the isobutanol present in the solution on a boiling water-bath for about 15 min. in an open beaker. Some isobutanol is still left in the solution, but as it does not interfere with the final estimation, its complete removal is not necessary. Add to the hot solution 15 ml. of distilled water, and cool it down to about 40–50°. Acidify further with 6 drops of concentrated HCl and oxidize by adding a 4% solution of $KMnO_4$ drop by drop with continuous stirring, until the urine is completely decolorized and the pink colour of the permanganate stays for more than 30 sec. Allow the solution to cool. Stand for about 15 min. to ensure that any excess of $KMnO_4$ is completely reduced, then neutralize cautiously with 40% NaOH to pH 6·5–6·8 with bromothymol blue as external indicator. It is essential that the pH 6·8 should not be overstepped, as an alkaline reaction causes a brownish coloration. Make up to 50 ml. with distilled water and centrifuge off any precipitate that is formed.

(2) *Reaction with* CNBr. Four graduated 15 ml. flasks are labelled A, B, C and D. To flasks C and D add 0·5 ml. of diluted standard nicotinic acid solution. To flask A add 0·6 ml. of 10% HCl; this flask is to be used as a blank. Measure 10 ml. of the urine solution into each of the four flasks. Mix well, especially flask A, then add to all four flasks 2 ml. of CNBr solution. After the mixing, place the flasks for 4 min. in a water-bath kept at 56–58°. Cool the flasks for 5 min. in tap water (temperature 18–20°) in a darkened place.

(3) *Development of colour.* To each flask add 1 ml. of the alcoholic amine reagent and shake. Then to flask B, C and D add 0·6 ml. of 10% HCl. Fill the flasks up to the 15 ml. mark with distilled water. Mix the contents and allow to stand for 5 min. in a darkened place. Immediately afterwards take measurements with the Pulfrich photometer, using 3 cm. cells and filter S47 [Kodicek, 1940*a*, *b*]. Flask A is used as the blank, and the flasks B, C and D are examined for their contents of nicotinic acid.

(4) *Calculation of result.* The relation between intensity of colour (extinction) and nicotinic acid present is linear [Harris & Raymond, 1939]. Hence, nicotinic acid content, μg./ml. of urine, equals

$$\frac{B-A}{(C-A)-(B-A)} \times S \times \frac{F}{10} \times 1{\cdot}1 \times \frac{25}{20} \times \frac{1}{V_0}$$
$$= \frac{B-A}{C-B} \times S \times \frac{F}{V_0} \times 1{\cdot}375.$$

When, according to the procedure, $S = 10$ μg., $F = 50$ ml. and $V_0 = 20$ ml., the formula is simplified to $\frac{B-A}{C-B} \times 3{\cdot}44$ μg./ml. of urine. A = extinction of solution in flask A (blank), B = extinction of solution in flask B (urine), C = mean of extinctions of solution in flasks C and D (urine + added nicotinic acid), S = μg. of nicotinic acid added to flasks C and D, F = final volume of urine after washing with isobutanol, oxidation and neutralization, V_0 = original volume of urine taken for analysis, 1·1 = factor allowing for the increase in volume of washed urine due to isobutanol.

Reproducibility of method

To test the reproducibility of the method separate determinations were done on one specimen of urine. The results are shown in Table 11. The standard error of a single determination is about ±2·4%. This degree of precision is highly satisfactory.

Table 11. *Data testing the reproducibility of method*

Exp. no.	Nicotinic acid in same specimen of urine (μg./ml.)
1	0·81
2	0·86
3	0·85
4	0·82
5	0·81
Average	0·83

Standard deviation of single determination ±0·02 (2·4%).

RESULTS

Daily excretion of nicotinic acid by normal individuals determined by the new procedure

Ten normal subjects were examined for their daily excretion of nicotinic acid under standard conditions (see below). The final modification described above was used. The resting levels of adults varied from 0·85 to 2·33 mg. of nicotinic acid/day, with an average of 1·15 mg. (Table 12). These figures agree with the values found by the earlier procedure of charcoal decolorization.

Table 12. *Daily excretion of nicotinic acid by normal individuals as determined by the final modification*

Subject	Age	Sex	Daily volume of urine (ml.)	Nicotinic acid excreted (mg./day)
J.E.P.	17	F	880	0·85
E.M.C.	41	F	1450	1·03
J.Y.	30	M	1360	1·14
L.W.M.	33	M	1400	1·16
A.G.D.	17	M	1040	1·43
E.K.	33	M	1100	1·53
S.G.I.	30	M	2160	1·77
T.M.	41	M	2350	1·92
D.W.	19	M	1630	2·33
J.K.	4	F	650	0·46

*Excretion after experimental dosing of healthy subjects with nicotinic acid**

Four normal subjects living on their usual diets were examined for their urinary excretion of nicotinic acid. The daily output of urine was collected in two portions, a morning 3 hr. sample and the remaining 21 hr. portion; each portion was analyzed separately. Test doses were taken with or after breakfast. Table 13 gives the results of the experiment.

The average daily excretions of nicotinic acid by the four individuals were 1·09, 1·42, 1·47 and 2·10 mg., and the corresponding morning 3-hourly outputs averaged 0·23, 0·09, 0·13 and 0·16 mg. respectively. Smoking did not appreciably change the values. After daily ingestion of 100 mg. of nicotinic acid for several days, the average daily excretions of the last three individuals increased to 7·77, 3·98 and 9·51 mg., and the corresponding 3-hourly excretions to 5·34, 2·02 and 6·38 mg. respectively.

It will be seen from Table 13 that while the excretions of nicotinic acid by the different subjects on their usual diets changed very little from day to day and varied little from one to another, the outputs after ingestion of test doses fluctuated considerably,

* Decolorization of urine in these experiments had been performed by charcoal adsorption as described in § II(*a*). Urine collected after dosing had, however, such a high concentration of nicotinic acid that the charcoal adsorption could be replaced by a simple dilution of the urine.

Table 13. *Response of normal individuals to test doses of nicotinic acid*

Subject	Day	Test dose (mg.)		Nicotinic acid excreted (mg.) in 3 hr.	Nicotinic acid excreted (mg.) in 24 hr.	Notes
Y.L.W.	1	0		—	2·24	No smoking
Body weight	2	0		—	2·21	,,
65 kg.	3	0		—	1·56	,,
	4	0		—	1·26	Smoking
	5	0		0·05	1·21	,,
	6	0		0·12	0·71	,,
	7	0		0·10	1·40	,,
	8	0		0·08	0·77	,,
			Average	0·09	1·42	
	9	100		2·91	4·68	
	10	100		5·16	8·38	
	11	100		2·34	3·74	
	12	100		2·98	4·92	
	13	100		16·30	21·20	
	14	100		2·32	3·70	
			Average	5·34	7·77	
L.J.H.	1	0		—	0·63	No smoking
Body weight	2	0		—	2·21	,,
72 kg.	3	0		—	2·16	,,
	4	0		—	2·56	Smoking
	5	0		—	1·90	,,
	6	0		0·14	2·06	,,
	7	0		0·12	1·25	,,
	8	0		0·17	1·09	,,
	9	0		0·20	1·91	,,
			Average	0·16	2·10	
	10	100		1·33	3·07	
	11	100		5·16	7·82	
	12	100		4·48	7·60	
	13	100		17·50	23·98	Dose taken on empty stomach
	14	100		2·00	4·28	
	15	100		7·82	10·30	
			Average	6·38	9·51	
	16	0		0·53	2·30	
	17	0		0·17	1·27	
	18	0		0·59	4·39	
E.K.	1	0		—	2·50	No smoking
Body weight	2	0		—	1·85	,,
87 kg.	3	0		—	1·37	,,
	4	0		—	1·12	Smoking
	5	0		—	1·77	,,
	6	0		0·10	0·82	,,
	7	0		0·15	1·00	,,
	8	0		0·14	1·48	,,
	9	0		0·14	1·28	,,
			Average	0·13	1·47	
	10	100		1·13	2·58	
	11	100		1·75	2·98	
	12	100		1·49	2·47	
	13	100		0·61	2·32	
	14	100		4·60	9·20	
	15	100		2·24	3·81	
	16	100		2·35	4·47	
			Average	2·02	3·98	
	17	0		1·22	3·08	
	18	0		0·11	2·53	
G.G.	1	0		—	1·55	
Body weight	2	0		—	0·71	
83 kg.	3	0		—	0·92	
	4	0		—	0·91	
	5	0		—	0·77	
	6	0		0·16	0·71	
	7	0		0·10	0·86	
	8	0		0·42	1·55	
	9	0		0·24	1·85	
			Average	0·23	1·09	

making it difficult to draw any definite conclusion from the experiment. It will be noted also that the different individuals varied a great deal in their response to dosing. Immediately after the cessation of dosing, the excretion of nicotinic acid returned to the previous level.

In view of the big fluctuations in excretion after the intake of test doses, it was thought advisable to standardize the conditions under which the doses were administered. Three subjects were given a special breakfast which contained little nicotinic acid or trigonelline, and consisted of two cups of tea, two slices of white bread, jam, butter and about 50 ml. of milk, and the dose given with the breakfast after one cup of tea and one slice of bread. The urines were collected and analyzed as before, and the results are given in Table 14. It will be seen that the 3-hourly excretions after dosing were fairly constant from day to day, and also the 24 hr. outputs did not fluctuate as before when the conditions of dosing were not standardized. It is obvious that if test doses of nicotinic acid are to be used for assessing the body reserves of the vitamin, they must be administered under a standardized condition, otherwise little information can be gained from the results.

Time curve of excretion of ingested nicotinic acid under uniform conditions

In one normal subject the hourly excretion of nicotinic acid after ingestion of 100 mg. of nicotinic acid was studied under standardized conditions. The excretion was followed at hourly intervals for 5 hr. (Table 15). Our findings, similar to those of Bandier [1939*a*], were that 91 % of the administered nicotinic acid was excreted in the first 5 hr. after the dose, and that the maximum excretion occurred in the first hour and amounted to about 63 %. This rapid elimination suggests that the organism is not able to utilize efficiently all the ingested nicotinic acid and therefore excretes the greater part of it promptly. This phenomenon does not occur when our subjects are dosed with nicotinic amide. Both the acid and the amide, however, produce a large

Table 14. *Response of normal individuals under standardized conditions of dosing*

Subject	Day	Test dose of nicotinic acid (mg.)		Nicotinic acid excreted (mg.) in 3 hr.	Nicotinic acid excreted (mg.) in 24 hr.
Y.L.W.	1	0		0·12	1·20
Body weight	2	0		0·16	1·21
65 kg.			Average	0·14	1·21
	3	100		9·70	12·28
	4	100		11·20	13·80
	5	100		8·40	—
			Average	9·97	13·04
	6	0		0·21	6·61
	7	0		0·18	1·68
	8	0		0·15	0·90
E.K.	1	0		0·41	1·64
Body weight	2	0		0·11	1·40
87 kg.			Average	0·26	1·52
	3	100		0·85	2·36
	4	100		1·27	3·31
	5	100		1·03	11·03
			Average	1·05	5·57
	6	0		0·25	1·90
	7	0		0·25	1·99
	8	0		0·22	1·05
G.G.	1	0		0·16	0·71
Body weight	2	0		0·10	0·86
83 kg.	3	0		0·42	1·55
	4	0		0·24	1·85
			Average	0·23	1·26
	5	100		10·89	13·69
	6	100		9·85	11·17
	7	100		11·20	13·93
			Average	10·65	12·93
	8	0		0·20	1·40
	9	0		0·10	1·23

Table 15. *Rate of excretion of ingested nicotinic acid*

Time	Nicotinic acid excreted (mg./hr.)
3 hr. period before dosing	0·23
Total 24 hr. period before dosing	1·26
100 mg. nicotinic acid given by mouth	
0 hr.	—
1	7·77
2	2·46
3	0·66
4	0·37
5	0·25
5 hr. total excretion	11·51
5–24 hr.	2·18
24 hr. total excretion	13·69

increase in the excretion of trigonelline and related N-methyl pyridinium compounds which can be regarded as normal metabolites. The fate of ingested nicotinic acid or amide in the body has been briefly discussed in our previous report [Kodicek & Wang, 1941]. The detailed results will be published in a subsequent paper. On the basis of these findings, we have recommended dosing with nicotinic amide, followed by the estimation of the urinary excretion both of trigonelline and nicotinic acid, as a method of assessing the level of nutrition with regard to the anti-pellagra vitamin.

SUMMARY

1. The cyanogen bromide-*p*-aminoacetophenone method of Harris & Raymond for the estimation of nicotinic acid in urine has been further studied, and several additional improvements have been introduced, viz. (*a*) decolorization of the digested urine by washing with isobutanol and oxidation with $KMnO_4$; (*b*) the use of a lower temperature, a shorter time for the reaction of CNBr with nicotinic acid, and the addition of larger amounts of *p*-aminoacetophenone to the reaction mixture; and (*c*) the introduction of a modified blank.

2. The normal excretion of nicotinic acid by ten healthy subjects and the response to test doses of nicotinic acid by four of them has been studied.

3. The maximum excretion of ingested nicotinic acid occurs in the first three hours, the first hour showing the highest output.

4. It is emphasized that when test doses of nicotinic acid are used for assessing the body reserves of the vitamin, they should be administered under standardized conditions.

5. For obtaining reliable information of the nutritional status in regard to the anti-pellagra vitamin, dosing with nicotinic amide is to be preferred to dosing with nicotinic acid, and estimation should then be made of the excretion of trigonelline, as well as that of other substances related to nicotinic acid.

We wish to take this opportunity to thank Dr L. J. Harris for his constant interest and valuable advice; Mr G. G. Grindley for volunteering as an experimental subject, and Roche Products Ltd. for a gift of *p*-aminoacetophenone.

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The Etherification of Hydroxyamino-acid Residues in Silk Fibroin by Dimethyl Sulphate

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In recent years it has been recognized that in addition to tyrosine the hydroxyamino-acids serine and threonine are major protein constituents. The mode of linkage of the —OH groups of these latter in the protein is of interest, since such groups might play a part in non-peptide linkage of amino-acid residues

[Chibnall, 1942; Linderstrøm-Lang & Jacobsen, 1941], or, if free, influence significantly the chemical and physical properties of the protein. Colour reactions and spectroscopy have thrown considerable light on the mode of incorporation in proteins of the phenolic —OH groups of tyrosine, but these methods are not of value in studying aliphatic —OH groups. The acylation of proteins has been studied by numerous workers, but, while the number and lability of acyl groups introduced provides circumstantial evidence for the occurrence of free —OH groups, the lability of *O*-acyl groups to the usual hydrolytic agents has precluded confirmation of this by the isolation of *O*-acylated compounds from hydrolysates of acylated proteins.

Methylation has been extensively and successfully employed for investigating the mode of linkage of aliphatic —OH groups in carbohydrates. Many workers, for a variety of purposes, have applied methylating agents to proteins [Blackburn, Carter & Phillips, 1941], but few [e.g. Skraup & Krause, 1909; Haurowitz, 1938] have employed conditions suitable for methylating aliphatic —OH groups, and of these none seems to have identified *O*-methylated compounds after hydrolyzing the protein. The strongly alkaline aqueous conditions, which appear necessary for the methylation of aliphatic —OH groups in proteins, induce degradative changes in the protein, making interpretation more complicated. Nevertheless, it seemed worth attempting the application of this technique in the protein field.

We have studied the action of dimethyl sulphate on silk fibroin in the presence of excess cold aqueous NaOH. This protein was chosen because of its resistance to solution by cold alkaline reagents, which facilitates repeated treatment, its negligible S content and its high content of hydroxyamino-acids.

Repeated treatment of silk fibroin in this way introduces *O*-methyl groups to an extent which cannot be increased by continued treatment. At this stage nearly all the tyrosine residues and about one-half the serine residues have been converted to their *O*-methyl derivatives. Our evidence for this is: (1) Disappearance of those colour reactions (Millon, xanthroproteic) due to free —OH groups of tyrosine residues from methylated fibroin and its hydrolysates. (2) Diminution of the hydroxyamino-acid content (NH_3 liberated by periodate) of hydrolysates of methylated fibroin to about one-half of the corresponding value for normal fibroin. (*O*-methylserine does not give NH_3 with periodate.) (3) The —OMe content of methylated fibroin and its hydrolysates corresponds stoichiometrically to the tyrosine + half the hydroxyamino-acid content of fibroin. (4) The occurrence of *O*-methyl-tyrosine and *O*-methyl-serine in hydrolysates of methylated fibroin. These have been demonstrated in the expected quantities, after conversion to their *N*-acetyl derivatives. For the latter compound we have employed partition chromatography on silica gel [cf. Gordon, Martin & Synge, 1943*a*].

Preliminary acetylation of the fibroin by boiling acetic anhydride [cf. Carr, 1938*b*] was found not to increase the amount or rapidity of methylation. Preliminary acetylation has been found useful in the methylation of polysaccharides [cf. Hirst & Young, 1939].

It was found that fully methylated fibroin on subsequent acetylation took up correspondingly fewer *O*-acetyl groups (determined by saponification with cold alkali) than normal fibroin. However, some acetyl groups saponifiable under these conditions were obtained on acetylation of fully methylated fibroin.

From the above it is apparent that the reaction of dimethyl sulphate with silk fibroin in the presence of alkali is entirely different from that occurring in its absence, which, according to Blackburn *et al.* [1941], leads to the introduction mainly of ester-type *O*-methyl which can be entirely liberated by the action of dilute alkali. Rutherford, Patterson & Harris [1940], by exhaustive treatment of fibroin with diazomethane in ether, obtained a product in which (as judged by non-isolative procedures) the tyrosine-phenolic groups were completely etherified, but there was no evidence for methylation of aliphatic —OH groups, most of the extra *O*-methyl being of a type similar to that studied by Blackburn *et al.* [1941].

Since the amino-N content of a hydrolysate of our fully methylated fibroin was little different from that of a hydrolysate of untreated fibroin, it seems unlikely that the reagents employed gave rise to significant *N*-methylation of amino-acid residues in peptide linkage.

Since the positions in the fibroin at which *O*-methyl groups are introduced by methylation appear also to be accessible to acetylation by a procedure [cf. Carr, 1938*b*] that does not seriously degrade the fibroin, it is reasonable to conclude that these (i.e. the —OH groups of nearly all the tyrosine and about one-half of the serine residues) are free in intact fibroin. For the tyrosine, this conclusion is consistent with the phenolic colour reactions of fibroin, and with the data of Rutherford *et al.* [1940] on methylation, of Meyer, Fuld & Klemm [1940] on coupling with diazo compounds, and of Kise & Carr [1936] and Carr [1938*a*, *b*] on acetylation. Serine residues having free —OH groups might be expected to take part in the alkaline degradation and coupling reactions demonstrated in whole silk by Nicolet & Shinn [1941 *a*, *b*].

The mode of linkage of the serine residues which do not undergo *O*-methylation remains obscure. The present work has not established whether or

not their —OH groups are accessible to acetylation. It is possible that they are not methylated owing to incorporation in 'crystallites' in the fibre, while serine residues in the 'encrusting substance' are reactive [cf. Meyer *et al.*, 1940; Synge, 1943; Huggins, 1943]. Or they might be involved in oxazoline rings of the type postulated by Linderstrøm-Lang & Jacobsen [1941] [cf. Blackburn, Middlebrook & Phillips, 1942]. The present work only demonstrates that the serine residues of silk fibroin fall into two distinct categories with respect to their reactivity with dimethyl sulphate and alkali in the cold. Investigation of the behaviour under these conditions of other proteins and model substances, though technically more difficult owing to their solubilities, may help to elucidate this question.

EXPERIMENTAL

Analytical methods

Estimation of tyrosine. The colorimetric method of Arnow [Block, 1938] was employed.

Estimation of total hydroxyamino-acids. The method of Van Slyke, Hiller & MacFadyen [1941] was modified, by the use throughout of one-tenth of the amounts of reagents which they employed, and by omission of the capryl alcohol. The reagents were added to the outer compartment of the Conway micro-diffusion apparatus. The NH_3 evolved after $1\frac{1}{4}$ hr. gentle rotatory shaking was determined by absorption in 1·0 ml. of 1% boric acid mixture, according to the method of Conway & O'Malley [1942]. Under these conditions we obtained 94–95% recovery of NH_3 (80–120 μg.) from ammonium sulphate in 2 ml. half-saturated K_2CO_3 solution. All results have been corrected for this incomplete absorption. Serine under these conditions yielded the theoretical quantity of NH_3.

Estimation of —OMe. On methylated fibroin and its hydrolysates this was determined by Dr S. Blackburn with the volatile iodide procedure of Baernstein [cf. Blackburn *et al.*, 1941]. Other determinations were made with the standard Pregl procedure by Dr G. Weiler, Oxford, who also carried out carbon and hydrogen determinations.

Estimation of total N. Semimicro-Kjeldahl determinations were employed, using the incineration mixture of Campbell & Hanna [1937].

Estimation of amino-N. The manometric Van Slyke procedure (4 min. reaction time) was employed. Where the sample contained free mineral acid, this was neutralized before analysis with excess of sodium acetate.

Melting points are given uncorrected. Optical rotations were observed in a 2 dm. tube.

Material

Material from the same batch of unweighted silk fabric as was studied by Blackburn *et al.* [1941] was employed throughout this work. The fabric was thoroughly rinsed with cold distilled water before use. Weights were determined after samples had been dried to constant weight over H_2SO_4 at room temp. *in vacuo*.

Methylation of fibroin

A typical experiment is described: 3·284 g. of fabric were cut into small pieces and placed in a bottle with 40 ml. of *N* NaOH solution. 0·75 ml. of dimethyl sulphate was added. At 10 min. intervals 20 ml. *N* NaOH solution and 0·75 ml. dimethyl sulphate were added, until altogether 7·5 ml. dimethyl sulphate had been added. The preparation was shaken gently at room temperature the whole time.

At the end, most of the liquor was poured from the fabric, and treatment was repeated. After each treatment a test portion of the fabric was placed in sodium nitrite-acetic acid solution, and the development of yellow colour was observed. This steadily decreased until after four treatments it was practically absent.

When the preparation had to be left between treatments, the fabric was suspended in dilute acetic acid.

Samples removed for determination of —OMe were washed in six changes of distilled water before analysis. The course of methylation is shown in Table 1.

Table 1. *Course of methylation of silk fibroin*

No. of treatments	*O*-methyl content (CH_3 as percentage of dry wt. of product)
4	1·95
6	2·09
9	2·28
12	2·15

It appears that *O*-methylation is substantially complete after six treatments. The weight of final product (12 treatments), together with that of samples removed during methylation, was 2·96 g. The original material had N, 18·3%, and the final product N, 17·6%. Recovery, based on the N content, was therefore about 85%.

After treatment the fabric was readily creased, had much lower tensile strength, and lacked lustre.

Methylation following acetylation of fibroin

Dry fibroin was acetylated by refluxing in excess of acetic anhydride for $\frac{1}{2}$ hr. The reagent was then washed off with water, and the material subjected to a single methylation treatment as above. The material was then rinsed, dried, and again subjected to acetylation. After four such cycles the product had *O*-methyl (CH_3 as percentage of dry weight) 1·64%, suggesting that the method has no advantages over direct methylation, especially since greater losses resulted.

Acetylation of normal and methylated fibroin

The fabrics were acetylated by refluxing for $\frac{1}{2}$ hr. in acetic anhydride, rinsed off with several changes of distilled water and dried.

The difference between the alkali neutralized by acetylated and unacetylated material on suspending in cold dilute alkali served as a measure of the *O*-acetyl groups introduced.

Approximately 60 mg. of fabric were placed in a stoppered flask in 10 ml. *N*/50 $Ba(OH)_2$, and kept at room temperature with occasional gentle agitation. Control experiments showed that under these conditions no further alkali was neutralized after 6 hr. After 24 hr. a measured portion of fluid was removed from the flask and titrated with dilute HCl, with the exclusion of CO_2 and with phenolphthalein as indicator. Calculated for the whole volume, the difference from the direct titration of the alkali gave the total base bound by the saponified fibroin together with that neutralized by the acetic acid liberated in the saponification. The results of these experiments are shown in Table 2.

Table 2. *Analyses of silk fibroin after various treatments*

Material	Equivalents expressed in terms of N (1 atom) as a percentage of total N of material analyzed: Untreated material	Same acetylated	Methylated (12 treatments)	Same acetylated
Alkali neutralized	5·1	18·4	1·9	6·0
O-methyl content	0·0	0·0	11·4	12·4

Thus for unmethylated fibroin the *O*-acetyl groups introduced are 18·4 − 5·1 = 13·3 % (equiv. in terms of N as a percentage of total N of fibroin), while for methylated fibroin the corresponding figure is 6·0 − 1·9 = 4·1 %. The difference of *O*-acetyl content (13·3 − 4·1 = 9·2 %) is in reasonable agreement with the *O*-methyl content of the methylated fibroin, shown in Table 2, where it is expressed in the same units. The decreased binding of alkali by methylated as compared with untreated fibroin is presumably due to methylation of the base-binding phenolic groups of tyrosine residues [Gleysteen & Harris, 1940].

The yields of insoluble material after methylation, acetylation and saponification, expressed on a N basis, are shown in Table 3.

Table 3. *Yields and analyses of fibroin after various treatments*

Treatment	Yield (total N of product as percentage of total N of untreated fibroin)	*O*-methyl content (—OMe (1 equiv.) expressed in terms of N (1 atom) as percentage of total N of product)
Untreated	100	—
Untreated after saponification	96·7	—
Methylated	86	11·4
Same material after saponification	83	12·6
Acetylated	97	—
Same material after saponification	85·5	—
Methylated and then acetylated	73·6	12·4
Same material after saponification	65·5	11·2

The relative constancy of the *O*-methyl content of the material (Table 3) despite the considerable losses encountered in these treatments suggests that these losses did not involve serious qualitative fractionation of the fibroin, and justifies the assumption that the material lost by solution during processing was not chemically very different from that retained.

Hydrolysis of methylated fibroin

Refluxing fully methylated fibroin for 24 hr. in 6 *N* HCl resulted in considerable de-methylation. Tyrosine colour reactions appeared in the hydrolysate. The —OMe content of the hydrolysate (determined after evaporation to dryness) was 2·3 % (—OMe expressed in terms of N (1 atom) as percentage of total N of material), compared with a figure of 11·4 % for the material before hydrolysis. The NH_3-N liberated by periodate was 10·0 % of the total N of the hydrolysate, the corresponding figure for a hydrolysate of untreated fibroin being 10·9 %.

Hydrolysis of methylated fibroin for 24 hr. in boiling 6 *N* NaOH reduced the —OMe content (same units—determined after neutralization of hydrolysate with HCl and evaporation to dryness) to 5·1 %. Destruction of *O*-methyl-tyrosine would not be expected under these conditions [Rutherford *et al.*, 1940], but when 35 mg. of *O*-methyl-*dl*-serine were refluxed for 24 hr. in excess 6 *N* NaOH with 500 mg. of untreated silk fibroin, only 26 %, as measured by an —OMe determination, remained.

Bell [1937] noted removal of ether-methyl during treatment of methylated glycogen with hot hydrochloric acid, and found that *p*-toluenesulphonic acid effected satisfactory hydrolysis without de-methylation. For the present work we have found sulphuric acid to be satisfactory. 70 % of the —OMe of 37 mg. *O*-methyl-*dl*-serine was recovered after refluxing with 200 mg. of untreated silk fibroin in excess 6 *N* H_2SO_4 for 24 hr. ($SO_4^{=}$ was removed from the hydrolysate exactly by precipitation with $BaCl_2$. The filtrate and washings were then evaporated to dryness *in vacuo* at room temperature before analysis.)

Analysis of hydrolysates of methylated and untreated fibroin

For comparative purposes, both methylated and untreated fibroins were hydrolyzed by refluxing for 24 hr. in 30 parts by weight of 6 *N* H_2SO_4. $SO_4^{=}$ was removed by addition of the calculated quantity of $BaCl_2$ in aqueous solution, and the filtrate and washings from $BaSO_4$ were evaporated to dryness *in vacuo* below 40° and made up to known volume in water before analysis. The results are given in Table 4.

Demonstration of O-methyl-tyrosine in hydrolysate of methylated fibroin

O-methyl-tyrosine was obtained as its *N*-acetyl derivative by direct extraction and crystallization after acetylation of the hydrolysate [cf. Synge, 1939].

Methylated fibroin containing 234 mg. N (OMe as N (1 atom) in % of total N, 11·4 %) was refluxed for 24 hr. in 25 ml. 6 *N* H_2SO_4. The resulting hydrolysate was neutralized with NaOH to thymol blue (yellow), concentrated *in vacuo* until crystallization set in, made alkaline by addition of further NaOH and acetylated at 0° by addition of 5 ml. of acetic anhydride and 50 ml. 2 *N* NaOH added in portions with shaking. After keeping alkaline 2–3 hr. the solution was acidified to thymol blue (pink) with 10 *N* H_2SO_4 and extracted five times with 125 ml. portions of B.P. chloroform. The combined chloroform extracts were evaporated to dryness. Crystallization of the residue from water yielded gummy crystals, which were freed from gum by two recrystallizations from chloroform. The final product (61 mg.) had m.p. 147°, not depressed on admixture of authentic *N*-acetyl-*O*-methyl-*l*-tyrosine, $[\alpha]_D^{20°} + 51·5°$ (ethanol, $c = 0·9$). Synge [1939] records for *N*-acetyl-*O*-methyl-*l*-tyrosine m.p. 151°, $[\alpha]_D^{20°} + 54·3°$, $+54·4°$ (ethanol, $c = 1·3$). (Found: C, 61·0; H, 6·53; N, 4·8; OMe, 12·4 %; acid equiv. wt. 250. Calc. for $C_{12}H_{15}O_4N$: C, 60·8; H, 6·33; N, 5·9; OMe, 13·1 %; acid equiv. wt. 237.)

Table 4. *Analytical data on hydrolysates of methylated and untreated fibroins*

	Percentage of total N of material analyzed			
	Methylated fibroin	Untreated fibroin		Reference
		Present work	Other authors	
	Before hydrolysis			
—OMe (1 equiv. = 1 atom N)	10·3	—	—	
	After hydrolysis			
(1) —OMe (1 equiv. = 1 atom N)	9·15	—	—	
(2) Amino-N	90	92	—	
(3) Tyrosine N	0·35	4·5	4·6*	Meyer, Fuld & Klemm [1940]
(4) NH_3-N + NH_3-N liberated by periodate	7·0	11·9	—	
(5) NH_3-N	1·45	1·0	—	
(6) Hydroxyamino-acid N (by difference of (4) and (5))	5·55	10·9	10·8*	Nicolet & Saidel [1941]

* N content of the fibroin analyzed by these workers assumed to be 18·3 %.

Demonstration of O-methyl-serine in hydrolysate of methylated fibroin

O-methyl-serine was separated as its *N*-acetyl derivative by partition chromatography on silica gel [Gordon *et al.*, 1943*a*]. Control experiments with authentic *N*-acetyl-*O*-methyl-*dl*-serine [Synge, 1939] showed that on partition chromatograms employing B.P. chloroform, ethyl acetate or 30 % propanol-cyclohexane as developing solvent, it is not separable from acetylalanine. With 17 % butanol-chloroform, *N*-acetyl-*O*-methyl-serine travels as a band sufficiently faster than acetylalanine to permit the recognition of its presence, though not fast enough to permit satisfactory separations. However, with 1 % butanol-chloroform, it moves as a separate band having *R* [Martin & Synge, 1941] approximately 1·5 times that of acetylalanine.

On the basis of this information the following experiments were carried out: Approximately 10 mg. samples of fully methylated (as used in previous section) and untreated fibroin were hydrolyzed with sulphuric acid, and the hydrolysates evaporated to dryness *in vacuo* below 40° after removal of $SO_4^{=}$ as described above. The hydrolysates were then analyzed according to the procedure given by Gordon *et al.* [1943*a*]. Pelargonin chloride was employed as indicator. During the development with 17 % butanol-chloroform it was noted that with the material from methylated, as compared with that from untreated fibroin, the bands corresponding to *N*-acetyl-tyrosine and *N*-acetyl-serine [Gordon, Martin & Synge, 1943*b*] were greatly reduced in intensity, while the band corresponding to acetylalanine was broader and exhibited a 'faint front', as with the synthetic mixtures of acetylalanine and *N*-acetyl-*O*-methyl-serine studied above. The bands corresponding to acetylglycine did not differ in appearance.

The values found for 'alanine' (N as percentage of total N of fibroin) were 24·5 % for untreated and 30·8 % for methylated fibroin. The difference (6·3 %) is consistent with the *O*-methyl-serine value suggested by the data given above. Bergmann & Niemann [1937] recorded for alanine in silk fibroin a corresponding figure of 21·8 %. They assumed their fibroin to contain 19·0 % N.

After titration the 'alanine' fractions were acidified and examined on 1 % butanol-chloroform chromatograms. That from methylated fibroin showed two bands, similar to those given by the synthetic mixtures described above, while that from untreated fibroin gave a single band (acetylalanine).

An attempt was then made to isolate *O*-methyl-serine as its acetyl derivative. Since the only authentic material available for comparison [Synge, 1939] was racemic, the acetylation was conducted under racemizing conditions [du Vigneaud & Meyer, 1932]. Complete racemization was not, however, achieved, and the crystallization of the final product may have been interfered with by partial racemization thus induced.

275 mg. of the same methylated fibroin were hydrolyzed with sulphuric acid, freed from $SO_4^{=}$ with $BaCl_2$ and evaporated to dryness as above. The product was treated with 5 ml. 2·4 *N* NaOH and 2 ml. acetic anhydride, the mixture being kept at 40° for 15 hr. The mixture was then cooled, made alkaline to thymolphthalein by addition of 12 ml. 3 *N* NaOH, and kept at room temperature for 2 hr. to saponify *O*-acetyl groups. The mixture was then acidified with 10 *N* H_2SO_4 and extracted with five 100 ml. portions of 17 % butanol-chloroform. The material in the combined extracts was subjected to chromatographic analysis with 17 % butanol-chloroform as developing solvent. The resulting 'alanine' band was subjected to further analysis, by development with 1 % butanol-chloroform. Of the two bands so obtained, the faster yielded 20 mg. of material. Treated with ethyl acetate-light petroleum this yielded 11 mg. of partly crystalline gummy material having m.p. 128–130°, $[\alpha]_D^{22°} - 12°$ (ethanol, $c = 0{\cdot}4$). The material depressed the m.p. of acetyl-*l*-alanine (m.p. 126°) and of acetyl-*dl*-alanine (m.p. 133°) on admixture by more than 30°. It proved difficult to recrystallize. OMe was determined on the material recovered from the polarimetric determination after removal of ethanol by evaporation *in vacuo* over H_2SO_4. (Found: OMe, 7·3 %. Calc. for $C_6H_{11}O_4N$: OMe, 19·3 %.)

SUMMARY

1. Repeated treatment of silk fibroin with dimethyl sulphate in *N* NaOH at room temperature results in *O*-methylation of nearly all its tyrosine residues and about one-half of its serine residues.

2. The groups accessible to etherification in this

way are also accessible to acetylation when the fibroin is treated with boiling acetic anhydride.

3. The existence is thus established of two categories of serine residue in silk fibroin, differing in their accessibility to etherification by dimethyl sulphate.

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The Relative Efficacy of Calcium Carbonate and Calcium Phosphate in Preventing Rickets in Rats

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Present knowledge of the metabolism of calcium and phosphorus suggests that calcium phosphate is likely to be more effective as a calcifying agent than the carbonate. Since the question of dietary supplies of calcium became topical with the proposal to add carbonate to bread, some experiments were begun to compare the power of the carbonate and the phosphate to prevent rickets in rats. The applicability of the findings to human rickets will be discussed after the presentation of the results.

EXPERIMENTAL

General plan. The general plan of the experiments was to place young rats on diets comprising mostly flour to which were added varying amounts of $CaCO_3$ or $CaHPO_4$; after 5 weeks on these diets the ash content of both femurs of each animal was determined. The rats at the beginning of the experiment were about 6 weeks old and weighed about 50 g.; they were kept in separate cages throughout the experiment and weighed weekly. Each group receiving a given level of Ca contained five animals. $CaCO_3$ was given as the preparation used to fortify bread, i.e. *creta preparata* B.P. The highest amount of each salt added to the diet was equivalent to 3% of Ca, i.e. 7·5% $CaCO_3$ or 10·1% $CaHPO_4$.

Diets. In most of the experiments the basal diet consisted of flour to which was added 1% NaCl. The flour was either white (70% extraction) or National Wheatmeal (85% extraction). When white flour was used the rats were given supplements of aneurin and riboflavin equivalent to 10μg. daily of the former and 4μg. of the latter. No supplement was given with the wheatmeal flour. For comparative purposes one experiment was carried out with a maize diet similar to that commonly used for producing rickets in rats. This basal diet contained maize meal 76 parts, gluten 20 parts.

To the basal flour or maize diets were added 1% NaCl and varying amounts of the calcium salts. No further additions were made. With three parts $CaCO_3$, the maize meal

diet is the rachitogenic diet 2965 of Steenbock & Black [1925] and is that normally used in this laboratory for producing rickets.

Examination of bones. The knee joints of the rats were X-rayed at the end of each experiment and in some instances during the course of the experiment. The femurs were removed on the day the rats were killed. They were cleaned by hand and extracted with ethanol in a Soxhlet apparatus, dried, weighed and ashed at dull-red heat. The results are expressed as the percentage of ash in the dried bones.

RESULTS

Effect of calcium carbonate and of calcium phosphate on the ash content of bones

(*a*) *White flour.* With diets containing no added Ca, the ash content of the bones was, in three experiments, between 32 and 35 % (Fig. 1). With added $CaHPO_4$, the maximal ash content of about 50 % was reached with approximately 0·4 % Ca

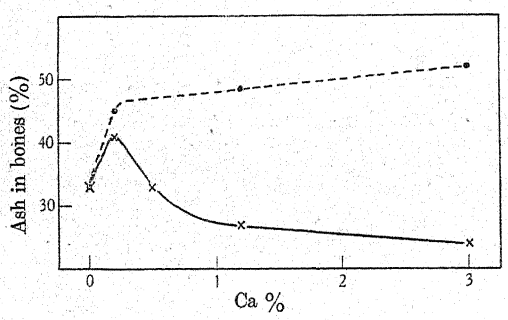

Fig. 1. White-flour diet: effect of $CaCO_3$ (continuous line) and $CaHPO_4$ (dotted line) on bone ash.

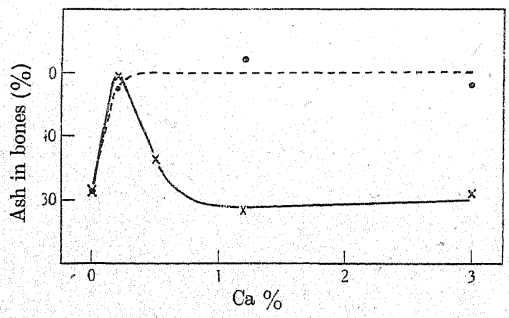

Fig. 2. Wheatmeal diet: effect of $CaCO_3$ and $CaHPO_4$ on bone ash.

and little difference was produced with an increase of Ca up to 3 %. With added $CaCO_3$, however, the ash content never exceeded 42 %, a level reached with 0·2 % Ca; higher levels of $CaCO_3$ reduced the ash content, which eventually fell to 28 %, i.e. below that of the animals receiving no added Ca. In a preliminary experiment the rats received a supplement of aneurin only and no riboflavin; although this influenced their growth rate (see below), it did not affect the ash content of the bones.

(*b*) *Wheatmeal flour.* In many respects, the experiments with wheatmeal flour (Fig. 2) gave results similar to those with white flour. The bones of animals on the basal diet with no added Ca had an ash content of about 32 %. The addition of $CaHPO_4$ to the diet produced an ash content of about 50 % with some 0·4 % Ca, and there was no change with an increasing amount of Ca. Addition of $CaCO_3$ resulted in a maximal ash content at about 0·2 % Ca, which rapidly fell with increasing amounts of dietary Ca to a value slightly below that of the animals on the basal diet. The only difference between the results with the white and with the wheatmeal flours appeared to be that the maximal calcification produced by the addition of $CaCO_3$ to wheatmeal flour was greater (about 50 %) than that produced with white flour (about 42 %); that is, with the former the maximal ash content was the same whether $CaCO_3$ or $CaHPO_4$ was added. Since, however, a slight difference in the amount of dietary $CaCO_3$ in the region of 0·2 % Ca produced such a great effect in the bones it is possible that an addition to white flour slightly higher or lower than this might also have resulted in an ash content approaching 50 %, and the apparent difference may be fortuitous.

(*c*) *Maize diet.* The results with the maize-gluten mixture were similar to those with flour (Fig. 3).

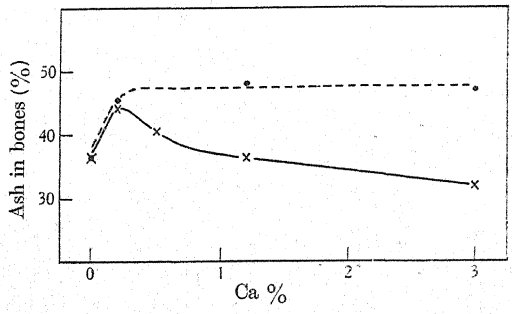

Fig. 3. Maize-gluten diet: effect of $CaCO_3$ and $CaHPO_4$ on bone ash.

The addition of $CaHPO_4$ in any concentration above 0·4 % Ca produced a high degree of calcification. Lower concentrations (0·2 % Ca) of $CaCO_3$ produced an improvement in calcification, but this deteriorated with higher concentrations. This diminution in effect was not so rapid as with the flour diets; for example, the ash content of the flour diets had reached its minimal level with 1·2 % Ca, whereas with the maize diet there was a further decrease from 37 % with 1·2 % Ca, to 32 % with 3 % Ca.

Effect of calcium carbonate and calcium phosphate on growth

It is known that, other things being equal, the degree of rickets which an animal develops is proportional to its rate of growth. It might have been thought that the differences in the calcification noted above were due to differences in rates of growth; the higher levels of dietary $CaCO_3$, for example, might be producing their rachitogenic effect by increasing the rate of growth. Such an explanation, however, must be discounted. A study of the growth curves shows that the rate of growth was lowered in each instance by the higher amounts of $CaCO_3$ in the diet (Figs. 4–6). Also the rate of growth on the flour diets was less with $CaCO_3$ than with the $CaHPO_4$, whilst the reverse was true with the maize-gluten diet; as we have seen, in all of these diets the phosphate at high levels of intake produced better calcification than the carbonate.

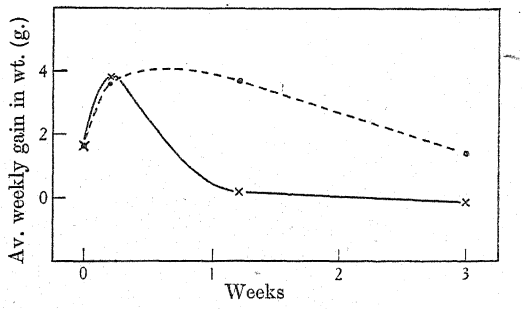

Fig. 4. White-flour diet: effect of $CaCO_3$ and $CaHPO_4$ on growth.

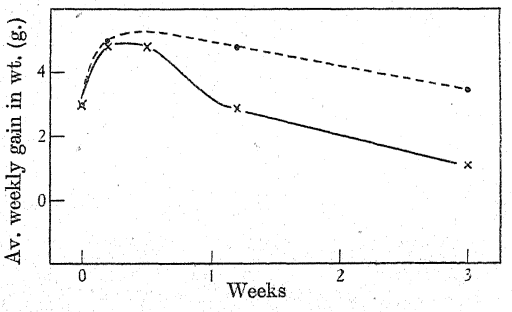

Fig. 5. Wheatmeal diet: effect of $CaCO_3$ and $CaHPO_4$ on growth.

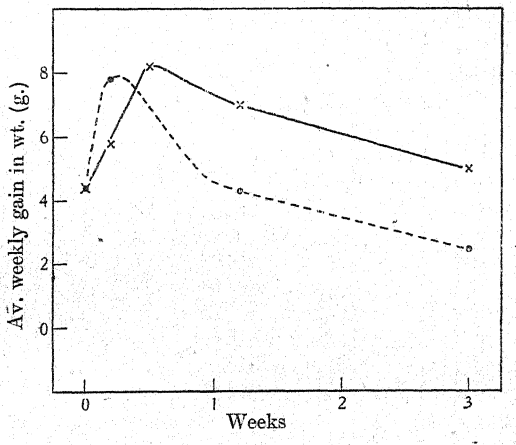

Fig. 6. Maize-gluten diet: effect of $CaCO_3$ and $CaHPO_4$ on growth.

There are other interesting features of the growth curves which are worth noting. Growth on the flour diets to some extent paralleled the ash content of the bones; this was especially noticeable with the white-flour diets containing $CaCO_3$. It was less true with those containing $CaHPO_4$, where the higher levels of this salt diminished growth, although they did not decrease the ash content of the bones. With the maize-gluten diet there was less parallelism; for example, with higher amounts of Ca, the $CaHPO_4$ resulted in a lower growth rate than $CaCO_3$, although it produced better calcification. In all instances growth was better on the wheatmeal-flour diet than on the white-flour diet, even though the latter contained added aneurin and riboflavin [cf. Chick, 1940]. In the preliminary experiments, to which reference has already been made, in which aneurin only was given, there was, in the absence of added Ca, a slight average loss of weight (0·7 g./week) compared with a gain of 1·7 g. when both vitamins were given. When 0·2 % Ca or more was added, either as $CaHPO_4$ or as $CaCO_3$, the growth-promoting effect of riboflavin was not observed.

DISCUSSION

It is known that the calcification of bone, especially in the absence of vitamin D, is to a large extent dependent on the Ca/P ratio in the diets. The different effects of the addition of $CaCO_3$ and $CaHPO_4$ to the diet on the bones of the animals appear to be due to this cause. In the absence of added Ca the ratio Ca/P is always low; 0·16 with white flour (70 % extraction), 0·10 with wheatmeal flour (unfortified; 85 % extraction) and 0·10 with the maize-gluten diet (Tables 1, 2). Addition of $CaHPO_4$ increases the ratio, which reaches a value of one, or slightly more, for all the diets, with an addition of 1·2 % Ca as $CaHPO_4$, and increases only slightly with further additions. With additions of $CaCO_3$ the ratio increases to very much higher levels. With 0·2 % Ca as $CaCO_3$, the ratio is 2·05 for white flour, 1·10 for wheatmeal flour and 1·00 for the maize-gluten diet; with higher levels of $CaCO_3$ the ratio rises rapidly, so that with 1·2 % Ca it is between 5 and 10 and with 3 % Ca it is between 13 and 28. In all instances, therefore, maximal calcification occurred with a ratio between 1 : 1 and 2 : 1.

Table 1. Ca *and* P *content of flours and maize-gluten mixture*

	Ca (mg./100 g.)	P (mg./100 mg.)	Ca/P
White flour	16·7	106	0·16
Wheatmeal flour	20	200	0·10
Maize-gluten mixture	22·3	221	0·10

These values have been very kindly supplied by Miss E. M. Widdowson. The values for the white flour and the maize-gluten mixture were from actual analyses of the samples used in these experiments; the values for the wheatmeal flour were average values obtained from the analysis of a large number of samples, in which it was found that the range of variation was fairly small.

Table 2. Ca/P *ratios of flours and maize-gluten mixture with various additions of* $CaCO_3$ *and* $CaHPO_4$

		Ca/P		
Addition to diet (g./100 g.)		White flour	Wheatmeal flour	Maize-gluten mixture
0		0·16	0·10	0·10
$CaCO_3$	0·05	0·64	0·35	0·33
	0·2	2·05	1·10	1·00
	0·5	4·87	2·60	2·36
	1·2	11·5	6·10	5·54
	3·0	28·5	15·1	13·7
$CaHPO_4$	0·05	0·46	0·29	0·28
	0·2	0·83	0·62	0·59
	1·2	1·17	1·08	1·06
	3·0	1·29	1·20	1·18

Adequate calcification depends not only on the ratio Ca/P but also on the absolute amounts of Ca and P; if both are low, the bones will not be well calcified. It may be that the poor calcification which occurred on the basal diets without added Ca was due to this fact and not to the very low Ca/P ratios. The amounts of P in the wheatmeal bread and maize-gluten diets are, however, fairly high. It seems likely therefore that the low ash content produced by the basal diets represents rickets of the 'low-calcium' type, whilst the low ash content produced by the higher levels of $CaCO_3$ represents rickets of the 'low-phosphorus' type. The mechanism of the production of 'low-phosphorus' rickets with high Ca/P ratios seems to be a reduction of the absorption of P by the excess of Ca.

It is recognized that the metabolism of Ca and P in human beings is appreciably different from that in rats. For example, rats usually absorb nearly 100 % of their dietary Ca, a proportion much higher than that ever reached in human subjects. Nevertheless, a comparison of rickets in human beings and in rats is not without some value. In both there is excessive faecal loss of $PO_4^{\equiv}$ and sometimes of Ca, as well as a low blood $PO_4^{\equiv}$. Administration of vitamin D in both cases restores these values to normal.

That $CaCO_3$ might be rachitogenic in human beings as well as in rats is suggested by experimental observations. McCance & Widdowson [1942] have demonstrated that absorption of $PO_4^{\equiv}$ in experimental subjects is lowered by the inclusion of $CaCO_3$ in bread. In experiments on babies and children, Stearns & Jeans [1934] showed that Ca and P were adequately retained in the body when given as milk or as $CaHPO_4$ but less was retained when Ca was given as carbonate, gluconate or lactate. Only one study, however, was made with $CaCO_3$.

The quantity of $CaCO_3$ at present added to wheatmeal bread is 65 mg. Ca to 100 g. flour (i.e. 0·065 %). In our experiments with rats these small amounts always result in improved calcification. It would, however, be unwise to transfer these quantitative results directly to the question of human rickets. Moreover, the rats were receiving diets restricted to a degree which would certainly not obtain in human subjects. The work here reported may best be considered as tests of the relative value of $CaCO_3$ and $CaHPO_4$ in favouring bone formation, using rats as a 'biological indicator'. The results suggest that at high levels of dosage the carbonate is inferior to the phosphate. At lower levels, comparable with the quantities added to bread, $CaCO_3$ is as efficacious as $CaHPO_4$ in improving the calcification of the bone of the rat. Whether the carbonate is as good as the phosphate for children, as obtains with growing rats, in the quantities likely to be ingested, can be determined only by trial.

SUMMARY

1. The effect has been studied in rats of the addition of calcium carbonate or calcium monohydrogen phosphate to basal diets consisting of white flour, wheatmeal flour or a maize-gluten mixture.

2. In all instances the addition to the diet of carbonate increased the ash content of the bones when it was added in small amounts in the proportion present in National Wheatmeal bread; larger amounts, however, led to a reduction in the ash content. Phosphate, on the other hand, produced adequate calcification, not only in these small amounts, but also in amounts up to 3 % Ca.

3. These differences were not due to the known aggravation of rickets which results from an acceleration of growth. With the white-flour diet, for example, the gain in weight was less with added carbonate than with phosphate.

4. The applicability of these results to human dietaries is discussed.

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The Rate of Enzymic Hydrolysis of Phosphoric Esters

2. RELATION OF STRUCTURE TO DISSOCIATION CONSTANT, MICHAELIS CONSTANT, AND RATE OF HYDROLYSIS

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Since Fischer [1898] suggested the possibility that a chemical combination took place between an enzyme and its substrate, the kinetics of many enzyme reactions have been explained by postulating such a combination and applying the mass action laws. The classical example of this form of treatment is the equation of Michaelis & Menten [1913]. In their study of mammary gland phosphatase, Folley & Kay [1935] obtained results which were in good agreement with the requirements of this equation, when allowance was made by the method of Haldane [1930] for inhibition with high substrate concentrations. Martland & Robison [1927] found no variation of activity of phosphatase with concentration, but our results suggest that these workers were using concentrations of substrate too high to show the Michaelis-Menten effect.

Several workers have noted the different rates at which phosphoric esters are hydrolyzed by phosphatase, e.g. Martland, Hansman & Robison [1924], Kay [1926; 1932] and Asakawa [1928]. King & Armstrong [1934] showed that hydrolysis of phenyl phosphate by bone phosphatase is more than twice as fast as that of glycerophosphate and many times faster than that of ethyl phosphate. King & Delory [1939] studied other phosphoric esters and suggested that the hydrolysis rates might be related to the Michaelis-Menten constants. In the present paper we have made a study of the rates of hydrolysis, optimum pH, and Michaelis constants, together with determination of the degree of dissociation of the phosphoric esters employed. The following esters were used: α-glycero-, β-glycero-, ethyl-, phenyl-, *o*-bromophenyl-, *m*-bromophenyl-, *p*-bromophenyl-, 2:4-dibromophenyl-, 2:4:6-tribromophenyl-, *p*-nitrophenyl-, and *o*-cresyl phosphates.

METHODS

Preparation of a potent phosphatase. A potent preparation was desirable in order to minimize the effect of contaminants and to permit short (5 min.) periods of hydrolysis, during which the rates of enzymic hydrolysis were not very different from initial velocities. Dialyzed and precipitated kidney and intestinal preparations were not effective. The potent phosphatase derived from dog faeces by the method of Armstrong [1935] was used. The brown powder obtained by procedures 'A' and 'B' of this author was dried *in vacuo*. In the presence of an optimal amount of Mg it showed an activity of 10,000 King-Armstrong phosphatase units/g., and still retained its activity after 5 years in the refrigerator.

A solution of 1 unit/4 ml. was prepared as follows: 1 mg. of the preparation was dissolved in 30 ml. 0·9 % NaCl containing enough $MgSO_4$ to make the final $MgSO_4$ concentration $M/100$. The phosphatase activity (about 30 units/100 ml.) was measured accurately by the method of King, Haslewood & Delory [1937]. The solution was then diluted so as to contain exactly 25 units/100 ml.

Preparation of phosphoric esters, see King & Nicholson [1939].

Determination of degree of dissociation of phosphoric esters. The apparatus used for the pH estimations was a hydrogen electrode with saturated calomel electrode and the Moloney [1921] platinum electrode. The latter was designed to hold a film of the solution, whose pH is to be determined, across the platinum electrode. Since it was only necessary for a small volume of solution to become saturated with H_2, ionic equilibrium was reached immediately. The potentiometer available was accurate to the nearest 0·01 pH. The apparatus was checked with a standard acetic acid-sodium acetate buffer of pH 4·63. The following procedure was adopted: 20 ml. of a $N/10$ solution of the salt of the phosphoric ester were pipetted into the reaction vessel. $N/10$ HCl was added in 0·2 ml. quantities, the pH being determined after each addition, and the pK determined graphically [cf. Pirie & Pinhey, 1929].

The graphs for *p*-bromophenyl-, *o*-bromophenyl-, phenyl- and α-glycero-phosphate are given as examples in Fig. 1. The pK's are included in Table 1.

Choice of a suitable buffer system. A buffer system covering the range of pH 7–11 and having the minimum effect on the rate of hydrolysis was obtained by combining the Michaelis [1930] veronal—HCl buffer and the Kolthoff [1925] Na_2CO_3-HCl buffer [King & Delory, 1940].

Determination of the optimum pH *for hydrolysis and rate of enzyme activity.* 4 ml. phosphatase solution (1 unit), 4 ml. buffer and 2 ml. $M/100$ substrate were incubated at 37° for exactly 5 min. The enzyme action was stopped by adding 2 ml. acid molybdate [King & Delory, 1939].

Determination of Michaelis constant:

$$\frac{v}{V}=\frac{x}{K_m+x}, \quad \text{or} \quad \log\frac{1}{x}=\log\frac{1}{K_m}+\log\left(\frac{V}{v}-1\right),$$

where x is the molar concentration of substrate, v is the velocity of hydrolysis, K_m is the dissociation constant of the enzyme-substrate compound (Michaelis constant) and V is the velocity when x is large compared with K_m. A curve of this equation will clearly be of the form of the dissociation of a weak acid and at the point at which $v/V=\frac{1}{2}$, i.e. where the velocity of hydrolysis is half the limiting velocity, K_m will be equal to the substrate concentration x.

Two ml. of the appropriate buffer solution together with an appropriate amount of water to make a final total volume of 10 ml. were pipetted into 15 ml. volumetric flasks. The flasks were placed in a water bath at 37° for 2 min. to allow the contents to attain the temperature of the bath. Two ml. of the enzyme solution were added, followed by the stated amount of $M/100$ substrate solution. The arrangement for the 7 flasks used was as follows:

Flask	ml. $M/100$ substrate	ml. water	log molarity of substrate
1	0·1	5·9	−4·00
2	0·2	5·8	−3·70
3	0·5	5·5	−3·30
4	1·0	5·0	−3·00
5	2·0	4·0	−2·70
6	4·0	2·0	−2·40
7	6·0	—	−2·23

After exactly 5 min., 2 ml. acid molybdate were added. An observer stood by with a stop-watch to note the time at which the substrate was added to each flask, and to signal when the enzyme activity should be stopped by the addition of the molybdate solution. 0·5 ml. aminonaphtholsulphonic acid was then added to each flask, and water to the mark [King, 1932]. The amount of inorganic P liberated was plotted against the logarithm of the substrate concentration. Inspection of the graph gave the substrate concentration at which half the limiting velocity was reached and the value was checked by substituting for several points in the Michaelis-Menten equation.

RESULTS

In Table 1 are given for each ester: (*a*) the negative logarithm of the dissociation constant (pK); (*b*) the pH at which hydrolysis by the faecal phosphatase

Table 1. *Rates of enzymic hydrolysis of phosphoric esters*

Phosphoric ester	pK	Optimum pH	Inorganic P enzymically liberated in 5 min. (mg./unit phosphatase)	K_m
p-bromophenyl	5·44	9·96	0·099	0·0003
2:4-dibromophenyl	5·60	9·95	0·085	0·0004
o-bromophenyl	5·60	9·80	0·086	0·0004
p-nitrophenyl	5·70	9·80	0·082	0·0005
phenyl	5·73	9·76	0·069	0·0006
o-cresyl	6·04	9·55	0·042	0·0008
2:4:6-tribromophenyl	6·10	8·94	0·036	0·0008
β-glycero	6·34	8·82	0·029	0·0012
α-glycero	6·44	8·62	0·019	0·0014
ethyl	6·45	8·08	0·008	0·0025
m-bromophenyl	Almost undissociated		Very little hydrolyzed	—

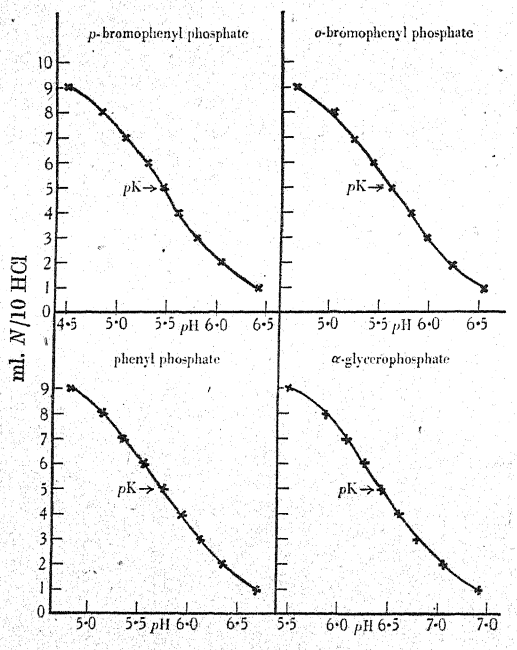

Fig. 1.

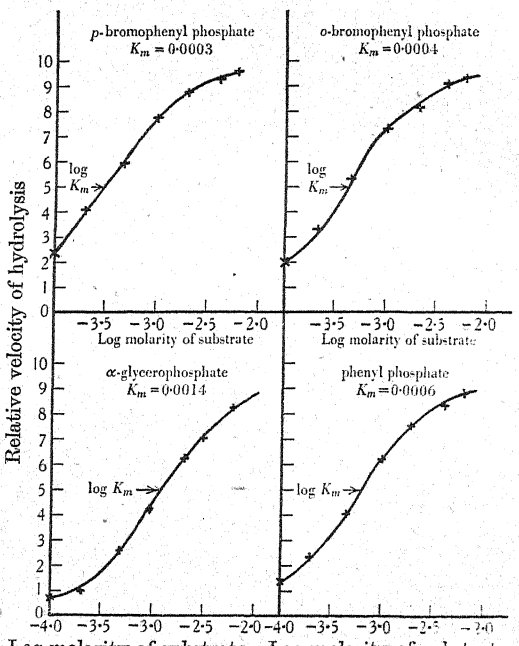

Fig. 2.

Fig. 1. Dissociation curves for phosphoric esters. 20 ml. of a $N/10$ solution of the salt of the ester titrated with $N/10$ HCl. Continuous lines were drawn from data derived from the equation $\mathrm{pH} = pK + \log \alpha/(1-\alpha)$, the value of the constant pK being chosen so as to give the best fit for the determined values.

Fig. 2. Curves showing Michaelis effect of phosphoric esters and faecal phosphatase at the optimum pH. The continuous lines were drawn from data derived from the equation $\log \frac{1}{x} = \log \frac{1}{K_m} + \log\left(\frac{V}{v} - 1\right)$, the values of the constant K_m being chosen so as to give the best fit for the determined values.

occurs at the maximum rate (optimum pH); and (*c*) the Michaelis constant (K_m) for the enzymic hydrolysis at the optimum pH. The pH-dissociation curves for *p*-bromophenyl-, *o*-bromophenyl-, phenyl- and α-glycero-phosphate are given in Fig. 1, while the curves showing the rate of enzymic hydrolysis at the optimum pH for the same four esters are illustrated in Fig. 2. In the latter case, the method adopted by Folley & Kay [1935] has been followed in the presentation of the graphs, the continuous line showing the theoretical curves for the Michaelis constant specified, and the experimental values being represented by crosses.

A preliminary experiment had shown that in the case of phenyl phosphate the theory of Michaelis was closely followed up to a concentration of 0·009 *M*, but that above this concentration, the velocity of hydrolysis decreased in accordance with the theory of Haldane [1930]. In the main series of experiments the higher concentrations, where deviations from the Michaelis-Menten equation might have been expected, were not investigated, since the main object was the determination of the Michaelis constants for the several esters.

The results indicate that with increasing acidity (i.e. decreasing pK) of the substrate there is an increase in the amount of hydrolysis, whilst the pH of optimum hydrolysis shifts to an increasingly alkaline reaction.

DISCUSSION

All the aromatic phosphoric esters (except *m*-bromophenyl) are more acidic and combine more strongly with the enzyme than the aliphatic esters. Of the latter ethyl phosphate is a weaker acid and is less strongly attacked enzymatically than the glycero-phosphates, which recalls the fact that ethyl chloride is much less chemically active than the chlorohydrins. The addition of an aliphatic group to an aromatic nucleus, as in *o*-cresyl phosphate, appreciably lowers activity.

The difference in behaviour of the *o*-, *m*-, and *p*-phenyl phosphates is noteworthy. The *p*-compound is the most rapidly hydrolyzed, whilst the *o*-compound occupies an intermediate position. These observations may be compared with the facts that *p*-methoxybenzyl bromide is hydrolyzed by acids much more rapidly than the benzyl bromide. The *o*-compound is also hydrolyzed more rapidly than the parent compound, whilst *m*-methoxybenzyl bromide is hydrolyzed less rapidly than the unsubstituted benzyl bromide [Waters, 1935]. It is also quite common to find a descending order of acidity in passing from *p*-compounds through *o*- to *m*-. An example may be given from the pK's of the nitrophenol isomers: *p*-nitrophenol 7·15, *o*-nitrophenol 7·17, *m*-nitrophenol 8·41.

Martland & Robison [1927] suggested two possibilities: (1) that phosphatase had the properties of a weak base whose undissociated molecules combine with a phosphoric ester to form a compound, which thereupon breaks down into the products of hydrolysis, or (2) that the enzyme combined with the ester to form a compound having the properties of a weak acid, whose anions suffer spontaneous decomposition. Our results seem to favour the first hypothesis, since an enzyme having basic properties might be expected to have the greatest affinity for that substrate with the most electro-negative grouping. Thus the greater the affinity between the substrate and the enzyme, the greater will be the rate of enzymic activity and the smaller the Michaelis constant.

Many workers, e.g. Martland & Robison [1927], have shown the gradual inactivation of phosphatase in alkaline solution. Kay [1932] has suggested that the rate of hydrolysis continues to increase with rise of alkalinity, and the apparent optimum is set by the increasing rate of enzyme destruction. This accords well with the further observation of Robison, who found that the enzymic hydrolysis of glycero-phosphate reached a maximum at pH 8·4 for an 18 hr. period of hydrolysis, and 9·4 for shorter periods. It is therefore suggested that the higher pH optimum values obtained here for the more acid esters are due to a greater protection of the enzyme against the inactivating effect of the hydroxyl ion. There are many examples of the protection of an enzyme by its substrate, e.g. trypsin is protected against heat inactivation by egg albumin, malt amylase by starch, and urea protects urease against the inactivating effect of certain dyestuffs.

The following explanation may, therefore, account for the results described in this communication. The enzyme has basic properties which enable it to combine with the substrate, the affinity between the two substances increasing with decreasing pK of the substrate. The greater this affinity, in other words, the smaller the Michaelis constant, the greater is the rate of hydrolysis, the greater is the degree of protection given to the enzyme against the inactivation effect of hydroxyl ions, and in consequence the higher the optimum pH.

SUMMARY

1. The rates of hydrolysis of several phosphoric esters by faecal phosphatase have been investigated, together with the Michaelis constants of the reactions and the dissociation constants of the esters.

2. The results show that with decreasing pK of the substrate the rate of hydrolysis increases progressively, and the enzyme is optimally active at a more alkaline pH. The Michaelis constants decrease with decreasing pK of the substrate.

3. These observations are consistent with the hypothesis of Martland & Robison [1927] that the enzyme possesses the properties of a weak base, whose undissociated molecule combines with the substrate to form a compound which breaks down into the products of hydrolysis, and with the postulate of Kay [1932] that the rate of hydrolysis continues to increase with increasing alkalinity and that the apparent optimum is set by the increasing rate of enzyme destruction.

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The Nutritive Value of Potato Protein for the Pig

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It has long been known that only about one-half of the nitrogen of the potato is in the form of protein, the remainder being in amino-acids and amides, chiefly asparagine. Neuberger & Sanger [1942] have recently shown that not only the total N of the potato, but also the relative proportions of the above three types of nitrogenous compounds, are widely different in different varieties, and that generally the amount of true protein N is rather less than half the total.

Although it is common convention in agricultural practice to reckon the protein value of the potato N as that obtained by adding together the true protein value and one-half of the value obtained by multiplying the non-protein N by 6·25, in this paper the proportion of the N in the different diets, rather than that of the protein, is considered, and the term crude protein is used to indicate the figure obtained by multiplying the total N by 6·25.

The maintenance of N equilibrium in adult man by potatoes was established by Hindhede [1913], Abderhalden, Ewald, Fodor & Rose [1915], Rose & Cooper [1917], Rubner & Thomas [1918] and by Kon & Klein [1928]. The last-named workers found that N equilibrium and good health could be maintained for several months in an adult man and woman on a diet of steamed potato, butter fat and fruit. The daily N intakes averaged 5·7 and 3·8 g. respectively.

The value of potato nitrogen for the support of growth. The value of potato N for the support of growth is not so clear, for it is difficult to obtain a sufficiency for young animals with potato alone since the protein is diluted with so much starch. McCollum, Simmonds & Parsons [1918] found that hardly any weight increase occurred in young rats when potatoes provided the only source of N. The diets contained but 7–8 % crude protein. However, growth was no better when cereal proteins were fed at the same level but was much improved on addition of 5 or 10 % of casein. Growth of rats on diets containing 9 % protein from oats, maize, peas or millet was much better and reproduction occurred when a proportion of these was substituted by potato, thus indicating a supplementary action of the N of the latter for that of the above grains [McCollum, Simmonds & Parsons, 1921]. Hartwell [1925; 1927] found that potato providing 7·6 % crude protein in the diet supported only very poor growth in rats and was quite insufficient for lactation.

Mitchell [1924*b*] determined the biological value of potato N for growth and maintenance in young rats by his modification [1924*a*] of Thomas's N balance technique. The biological value, when the diet contained 5 % crude protein, was 68·5, as compared with 72 for maize, 78·6 for oat, and 86·1 for rice protein. With 10 % crude protein in the diet, the biological values were 66·7 for potatoes, 59·6 for maize

and 64·9 for oats. These results are somewhat inconclusive, since potatoes appeared to be inferior to cereal proteins when the diet contained 5 % crude protein but as good or better when it contained 10 %.

Schiftan [1933] made a series of careful N balance experiments with pigs fed on diets in which respectively potatoes, barley and other foods were the only sources of protein. The tests were made with protein given at maintenance level, and also at a level to support both maintenance and growth. His experiments were carried out on four animals only and the collection periods were of 5 days' duration; his technique was, however, unexceptionable and good agreement was obtained in duplicate tests made on different pigs. For maintenance, the biological value of potato N in a diet containing 2·2 % crude protein was 69·6, and that of barley N in a diet containing 2·3 % was 58·7. Milk, casein (purified) and fish meal gave figures of 100·6, 69·3 and 66·2 respectively. For growth and maintenance, the biological value of potato N was 73·2 in a diet containing 13 % protein. The value for N of fishmeal was of about the same order and the values for that of milk and casein again superior. Potato N was thus slightly superior to that of barley in the tests at both low and high levels of intake. Since biological values of proteins normally fall as protein level is raised, one would expect that the value obtained for barley for growth and maintenance in the above experiment would be relatively too low in comparison with that given for potato. However, Schiftan, contrary to other investigators, found in general that his biological values increased slightly with level of protein fed.

Jones & Nelson [1931] obtained poor growth with rats on diets containing 9 % of crude protein from potato. Neither addition of gelatin, nor a supplement of tryptophan or of tyrosine and cystine with or without gelatin, improved matters, but addition of casein or lactalbumin supplemented the potato, and good growth occurred. The potato N was fed as a concentrate, prepared by removal of the starch, and contained 90 % of the original N. From this experiment it appeared that the potato lacked some unidentified essential amino-acid. Beadles, Braman & Mitchell [1930], on the other hand, also working with rats, had previously found that cystine itself improved growth when added to a potato diet containing 8–9 % crude protein. The potatoes were boiled and dried at low temperature.

The biological value of the true protein of potato appears to be fairly high. Kon [1928], using Mitchell's N balance technique for estimating its value for growth and maintenance of rats, found a biological value of 71 for tuberin compared with 68 for caseinogen; both proteins were fed at a level of 8·5–8·6 % in the diet. By the method of Osborne, Mendel & Ferry [1919] with growing rats, he obtained a maximum figure of 2·0 g. weight increase per g. of protein intake, when the diet contained 8 % protein.

Mangold & Columbus [1937] used Schiftan's technique to determine the biological value, for maintenance of N balance in pigs, of two dried potato protein concentrates called 'potato protein pulp flakes' (*Kartoffeleiweiss-pulpe-flocken*) and 'potato protein flakes' (*Kartoffeleiweiss-flocken*), respectively. In these preparations nearly all the N was in the form of true protein. The biological value of the 'potato protein pulp flakes' was 86·3 in a diet containing 3·8 % protein and 88·5 in one containing 7·1 %; that of the 'potato protein flakes' was 83·3 in a diet containing 5·1 % protein and 79·0 when it contained 9·7 %.

Digestibility of potato nitrogen. In the following paragraphs the coefficient of digestibility refers to 'apparent' digestibility, the figure for which is calculated from the difference between the N ingested and that eliminated in the faeces, on the assumption that the latter is derived from undigested food. The faeces, however, also contain N of endogenous origin, e.g. that contributed from gastric and intestinal secretions from, and into, the gut. A large proportion of the N of faeces is contained in its bacterial component and it is uncertain whether these bacteria are nourished from endogenous nitrogenous substances or from those of undigested food. The true digestibility of any food will therefore always be greater than the apparent digestibility.

The figures quoted in the literature for the digestibility of the crude protein of potatoes are very variable and usually low. Table 1 gives a summary of the figures obtained with swine for potatoes and various potato products; it will be seen that the coefficients of apparent digestibility vary from 0 to 84 %! There are four possible explanations of this great variation: (1) that the variations are due to differences in the samples or varieties of potatoes before cooking or processing, (2) that the digestibility is affected by processing, (3) that it depends on the bacterial flora inhabiting the gut, (4) that it is affected by other constituents of the ration. There is not sufficient evidence in the literature to permit discussion of the first and third possibilities, but so far as the effect of processing is concerned, it is noticeable that all the lower figures for digestibility with the pig have been obtained with potatoes dried in various ways. With herbivora, on the other hand, comparatively low digestibility coefficients have frequently been obtained for cooked fresh potatoes. For example, Woods [1919] found a coefficient of 43·4 % for the sheep, Brüggemann [1939] 58·3 % for the rabbit, and Woodward, Davidson, Watson, Robinson & Muir [1938] 65·0 % for the steer. Kellner, Just, Eisenkolbe & Poppe [1908] determined the digestibility of different dried potato products, fed in combination with soya-bean meal, with pigs and sheep; with both there were large differences between the different products, but the order of their merit was different in the two species. For example, potato flakes dried by the Paucksch process gave the highest coefficient (75·5 %) with pigs and the lowest (14·4 %) with sheep.

It is probable that some of the very low figures may be connected with the method of computation. In many investigations determination was first made of the digestibility of a ration, often of mixed foodstuffs, and then again when a portion had been substituted by potato or potato products. Any lowering in digestibility in the second case compared with the first was then attributed solely to the potato. If the potato was present in relatively small proportion, the result of such a calculation will give an unduly low figure for its digestibility.

Fingerling [1932] obtained the very low digestibility coefficient of 29·2 % for the crude protein of potato cossettes when fed to pigs with fishmeal and barley meal. When the basal ration consisted of fishmeal and potato flakes, the digestibility of the nitrogen of additional potato flakes, however, was 70·5 %. It is possible that in the first test the digestibility of the fishmeal and barley meal was depressed by addition of the potato cossettes and that, in the second, the addition of extra potato flakes caused no further depression in the digestibility of a ration which already contained them. With steers, the digestibility of the crude protein in both flakes and cossettes was low, but the order was reversed; the coefficient for flakes was negative while that for the cossettes was 30·9 %; in these experiments the

Table 1. *'Apparent' digestibility of the crude protein of potatoes for pigs*

Type of potato product	Digestibility coefficient of crude protein	Author
Raw	84·5	Snyder [1895]
Cooked	82·0	,,
Dried 'Kartoffelscheiben'	53·9	Kellner *et al.* [1908]
Dried 'Kartoffelschnitzel' (Schütz)	46·2	,,
Dried 'Kartoffelschnitzel' (Knauer)	63·5	,,
Dried 'Kartoffelschnitzel' (Petry & Kecking)	70·6	,,
Dried 'Kartoffelschnitzel' (Venuleth & Ellenberger)	42·9	,,
Dried 'Kartoffelflocken' (Paucksch)	75·5	,,
Dried 'Presskartoffel' (1)	26·7	Kellner & Neumann [1910]
Dried 'Presskartoffel' (2)	<0·0	,,
Dried 'Kartoffelflocken'	70·5	Fingerling [1932]
Dried 'Kartoffelschnitzel'	29·2	,,
Cooked	56·5*	Schiftan [1933]
Cooked	67·3*	,,
Dried 'Kartoffeltrockensaft-Pülpeflocken'	62·3	Nehring & Schramm [1937]
Dried 'Kartoffeleiweissflocken'	73·9	Mangold & Columbus [1937]
Dried 'Kartoffeleiweiss-Pulpe-Flocken'	66·5	,,
Cossette meal	39·9	Woodman & Evans [1939]

* True digestibility.

basal ration was composed of wheat straw, 'molasses slices' and cotton-seed meal. Fingerling notes in his paper that in the case of the steers the addition of potato products appeared to depress the digestibility of the N and N-free extract of the basal ration.

Woodman & Evans [1939] found the digestibility of the crude protein of potato cossettes, ground into a meal for pigs and sheep, to be 45·4 % for the sheep and 39·9 % for the pig. Tests *in vitro* gave a figure of 94 %. Here again it is possible that the apparent digestibility of the barley meal, weatings and white fish meal in the basal ration may have been lowered when fed with the potato cossette meal and thus a low estimate made of the digestibility of the latter.

The experiments of Kellner & Neumann [1910], on the other hand, gave little evidence that depression of the digestibility of the N of the constituents of the ration was caused by the presence of potato. They determined the digestibility of pressed dried potatoes, first when fed alone and later when fed with soya-bean meal. The coefficient of digestibility of the N of the potatoes fed alone was 26·7 %. Using this coefficient they calculated from the second experiment a coefficient for soya-bean meal of 94 %, a figure which could not have been so high if the pressed dried potatoes had appreciably lowered the digestibility of the soya-bean meal. However, from a further trial with a second sample of dried pressed potatoes, also fed with soya-bean meal, they calculated a negative digestibility for the crude protein of the potatoes when the figure of 94 % for the soya bean was used in the computation.

Section 1. GROWTH EXPERIMENTS

METHODS

The object of the experiment was to compare the growth of pigs which received all their protein as barley, or as barley supplemented with certain levels of casein, with that of pigs which received all their protein as potato, or as potato supplemented with identical levels of casein.

Mitchell's paired feeding method was adapted to the conditions of this experimental layout. Twenty-four pigs (17 hogs and 7 gilts) of the Wessex Saddleback breed were obtained from a local farm, and were kept in the same stalls and under the same system of management as those observed by Macrae, El-Sadr & Sellers [1942] in their studies on yeast protein. The animals were from four litters, all of which were farrowed between 6 and 16 January 1941; their individual identity was unknown. During the 3 weeks in which they were housed at the Institute prior to the commencement of the experiment, they were provided with a commercial pig food, with a few potatoes added daily. When they were 15–16 weeks of age, and of 25–36 kg. body weight, the groups and subgroups were selected. First, 12 pairs of as nearly equal live weights as possible were chosen: one pig from each of these pairs was allotted at random to a potato diet and the other to a barley diet, thus forming two groups (potato and barley), each comprising 12 animals. The four subgroups, each comprising three animals chosen at random from the potato group and a corresponding three animals from the barley group, received, respectively, unsupplemented diets, and diets containing 1, 3 and 6 % casein. All the pigs were individually fed in separate pens. Each of the 12 pigs receiving the potato diets consumed the same amounts of N and of dry matter as its corresponding barley-fed partner. The barley-fed pigs obtained slightly less digestible energy than the potato-fed animals because the barley was less digestible (see below), but there are not sufficiently reliable comparative figures to show whether this implied a lower intake of net energy under optimal conditions of protein intake.

The quantity of food given was regulated by the appetite of that pig of each pair which consumed the smaller amount: this was in every case the potato-fed pig. By this procedure, the results of the experiment could not be vitiated by

inability of the potato-fed pigs to consume as much of their more bulky ration as the barley-fed animals. It was not practicable to equalize the food intakes of animals receiving different levels of casein, since this would have meant reducing the food intake of all pigs to that of the one which ate least food. Except in so far as the food intake of the barley-fed animals was restricted by paired feeding, the diets were so constituted that the protein supply was the factor limiting growth. Only those pigs which received diets containing the highest proportion of N, viz. 2·4%, chiefly from casein, showed a growth rate approaching that obtained with Wessex Saddlebacks under good commercial conditions (H. A. Parsons, private communication; see Fig. 1).

The composition of the foodstuffs and of the experimental rations are set out in Tables 2, 3 and 4. In order to reduce the bulk of the potato diets, half the potatoes (on a dry matter basis) were given as boiled fresh potatoes and half as commercial potato flakes which were ground into a meal before mixing. Corresponding barley and potato rations were arranged to contain the same quantity of N (see Table 3).

The *cod-liver oil* was of the type used in veterinary practice, and was of guaranteed vitamin potency.

The *salt mixture*, made from commercial materials, contained bone ash 2, $CaCO_3$ 1, NaCl 1 and Fe_2O_3 0·1 parts.

The *yeast extract* was made by the method of Macrae *et al.* [1942], from brewer's yeast kindly supplied by Messrs Greene, King and Sons. The extract, which was protein-free, contained 1·607 mg. N/ml., and the daily dose, given separately with the ration of each pig, contained the equivalent of 30 g. dry yeast.

The *boiled potatoes* were from two batches of 'Majestic' potatoes, obtained locally and stored under straw in a tin hut; there was a good deal of sprouting during the experiment, but the sprouts were rubbed off, and the potatoes were washed before cooking. They were cooked in a cage lowered into a large copper of boiling water. The N content of the first batch was 1·58, and of the second, 1·85%, on a dry matter basis.

The *potato flakes* were obtained from the Farmers' Marketing and Supply Co., Ltd., and were processed at the beginning of March 1941. The processing was as follows: raw potatoes were thoroughly washed and cooked in a pressure steamer for approximately 20 min. The cooked potatoes were then passed through a mashing screw and spread over rollers heated by steam at a pressure of 80 lb./sq. in. The flakes contained 1·507% N on a dry matter basis.

The cooked potatoes were carefully mixed with the dry potato meal, starch, salts, cod-liver oil and water before feeding. It was necessary to feed the rations in a very sloppy state and to titivate the appetites of the animals with additions of water towards the end of each meal. The

Table 2. *Composition of foodstuffs incorporated in the diets*

	Dry matter (%)	Total N content of dry matter (%)	Gross energy (kg. cal./100 g. of dry matter)	True protein N (% of total N)	Non-protein N (% of total N)
Potatoes (Majestic): Batch 1	29·90*	1·570*	406·0	41·1	58·9
Batch 2	28·07†	1·848†	402·7	39·8	60·2
Casein	89·53	15·26	567·9	—	—
Potato flake meal	87·47	1·507	403·0	—	—
Barley meal (1)	84·31‡	1·826‡	418·9	—	—
(2)	85·30§	1·869§	418·9	—	—
Yeast extract	—	1·607 mg./ml.	—	—	—
Starch	87·00	—	422·0	—	—
Cod-liver oil	—	—	945·0	—	—

* Mean of 29 duplicate determinations.
† Mean of 27 duplicate determinations.
‡ Mean of 6 duplicate determinations.
§ Mean of 3 duplicate determinations.

Table 3. *Percentage composition of the diets, calculated on dry weights*

Subgroup	Boiled potatoes	Potato flake meal	Barley meal	Casein	Starch	Salt mixture	Cod-liver oil	N content of dry matter (%)
No casein: Barley	—	—	79·90	—	16·60	2·00	1·50	1·49
Potato	45·25	45·25	—	—	6·00	2·00	1·50	1·49
1% casein: Barley	—	—	79·90	1·00	15·60	2·00	1·50	1·64
Potato	45·25	45·25	—	1·00	5·00	2·00	1·50	1·64
3% casein: Barley	—	—	79·90	3·00	13·60	2·00	1·50	1·95
Potato	45·25	45·25	—	3·00	3·00	2·00	1·50	1·95
6% casein: Barley	—	—	79·90	6·00	10·60	2·00	1·50	2·40
Potato	45·25	45·25	—	6·00	—	2·00	1·50	2·40

In addition pigs received yeast extract equivalent to 30 g. dry yeast daily.

The following materials were utilized in the construction of the rations:

The *barley meal* was ground whole barley from a single batch.

The *casein* was 'Ashless G.L.', described by the manufacturers (Glaxo Laboratories, Ltd.) as a 'self-soured' type, well washed with dilute acetic acid. It contained 15·27% N on a dry matter basis.

The *starch* was purest maize starch, obtained from Messrs Mackean of Paisley; it contained about 0·04% N.

Table 4. *Analysis of diets*

Sub-group	Ration	% casein in diet	g. N/100 g. dry diet contributed by				% of total N contributed by				Total quantities/100 g.			
			Boiled potatoes	Flake potatoes	Barley meal	Casein	Boiled potatoes	Flake potatoes	Barley meal	Casein	N (g.)	Digestible N (g.)	Gross energy (kg. cal.)	Digestible energy (kg. cal.)
1	Barley	0	—	—	1·494	—	—	—	100·0	—	1·494	1·212	418·93	366·61
	Potato	0	0·813	0·681	—	—	54·4	45·6	—	—	1·494	1·242	401·08	382·25
2	Barley	1	—	—	1·494	0·151	—	—	90·8	9·2	1·645	1·341	420·30	358·59
	Potato	1	0·813	0·681	—	0·151	49·4	41·4	—	9·2	1·645	1·348	405·54	382·02
3	Barley	3	—	—	1·494	0·455	—	—	76·7	23·3	1·949	1·651	423·31	366·59
	Potato	3	0·813	0·681	—	0·455	41·8	34·9	—	23·3	1·949	1·671	408·46	385·18
4	Barley	6	—	—	1·494	0·909	—	—	62·2	37·8	2·403	2·048	427·68	360·53
	Potato	6	0·813	0·681	—	0·909	33·9	28·3	—	37·8	2·403	2·142	412·83	389·30

amounts of food left unconsumed by the potato-fed animals were often considerable, and it was then necessary to weigh the residues and to determine their water content.

The analyses of the materials are given in Table 2 and details of the composition of the different diets in Tables 3 and 4.

RESULTS

The experimental rations were fed for a preliminary period of a week before live weight increases were recorded. About three days after this feeding was commenced, scouring was noted in the potato-fed animals of each subgroup. This scouring reappeared at intervals throughout the experiment, but was never severe. Otherwise the pigs remained in good health. The condition of the coat remained good in all animals, and no skin lesions developed. Carriage and behaviour were in most cases normal throughout. Coprophagy was never observed. During the 7th week of the test there was an exceptional heat wave, and all the potato-fed animals went off their feed. Pigs 8, 10 and 12 were once observed to vomit and scoured at this time. In addition, pig 21, a barley-fed animal, went off its feed and vomited once. No stiffness of the legs was noticed until the last fortnight of the experiment, when a few of the heavier pigs were a little stiff. This was probably because bedding material was not provided. The joints of the animals did not show any macroscopic lesions on slaughter.

Fig. 1 gives the composite growth curves of the various subgroups; Table 5 gives details of their food intake and weight increases. In 11 of the 12 pairs of pigs, the barley-fed pig grew better. The total weight increase of the barley-fed pigs during the 12 weeks averaged 29·5 kg., while that of the potato-fed animals was 23·6 kg.; the weight increase per unit N intake and per unit digestible N intake was higher, and food intake and digestibility energy per unit weight increase were lower, in the barley-fed animals (Table 5).

The results of our experiment are not, therefore, in accordance with those of Schiftan, who found the N of fresh potatoes of higher biological value than that of barley. It is possible that the value of the N of the potato flakes used in our experiments was somewhat reduced by heat treatment during processing. The pigs in subgroup 1 which received the potato N without casein supplement, made better gains than those fed on maize without supplement in the experiment of Macrae *et al.* [1942]. The initial weights of the pigs in our experiment were, however, initially greater than those used by Macrae *et al.*, which averaged about 20 kg. They would therefore not require so large a proportion of N in their ration, since, as age and live weight increase, relatively more fat and less muscle are laid down.

A comparison of the figures obtained from the four subgroups, summarized in Table 5, shows that:

Table 5. *Experimental data for individual pigs during whole experimental period*

Sub-group	Casein in diet (%)	Pig No.	Pig Sex	Ration	Body weight: At beginning (kg.)	Body weight: At end (kg.)	Body weight: Increase (kg.)	Body weight: Average weekly increase (kg.)	Total dry food intake (kg.)	Total N intake (kg.)	Wt. increase/unit intake of N	Wt. increase/unit intake of digestible N	Food intake/unit wt. gained	Digestible energy consumed/unit wt. gain (kg. cal./kg.)
1	0	1	♂	Barley	34·6	69·9	35·3	2·94	128·25	1·893	18·7	22·9	3·63	13,300
		2	♂	Potato	34·5	66·4	31·9	2·66	129·83	1·931	16·5	19·9	4·07	15,600
		3	♂	Barley	35·8	58·6	22·8	1·90	94·21	1·399	16·3	20·0	4·13	15,100
		4	♂	Potato	35·2	51·6	16·4	1·37	87·44	1·416	11·6	13·9	5·33	22,300
		5	♀	Barley	32·0	49·0	17·0	1·42	77·83	1·158	14·7	18·1	4·58	16,800
		6	♂	Potato	31·5	42·0	10·5	0·88	79·13	1·174	8·9	10·7	7·54	28,900
		Average barley fed			—	—	25·0	2·1	—	—	16·6	20·3	4·11	15,100
		Average potato fed			—	—	19·6	1·6	—	—	12·3	14·8	5·65	22,300
2	1	13	♂	Barley	36·6	63·4	26·8	2·23	102·22	1·670	16·1	19·7	3·81	13,700
		14	♀	Potato	34·5	55·8	21·3	1·78	103·87	1·693	12·6	15·4	4·88	18,700
		15	♀	Barley	26·6	43·0	16·4	1·37	74·30	1·223	13·4	16·4	4·53	16,200
		16	♂	Potato	27·2	39·8	12·6	1·05	74·38	1·220	10·3	12·6	5·90	22,600
		17	♂	Barley	32·0	64·8	32·8	2·73	118·66	1·931	17·0	20·8	3·62	13,000
		18	♂	Potato	33·0	61·1	28·1	2·34	120·55	1·963	14·3	17·5	4·29	16,500
		Average barley fed			—	—	25·3	2·1	—	—	15·5	19·0	3·99	14,300
		Average potato fed			—	—	20·7	1·7	—	—	12·4	15·2	5·02	19,300
3	3	7	♀	Barley	33·8	65·2	31·4	2·62	93·14	1·808	17·4	20·5	2·97	10,900
		8	♂	Potato	34·6	54·7	20·1	1·68	95·10	1·835	11·0	12·8	4·73	18,300
		9	♂	Barley	32·4	60·6	28·2	2·35	95·73	1·859	15·2	17·9	3·39	12,400
		10	♀	Potato	32·7	54·2	21·5	1·79	92·54	1·781	12·1	14·1	4·30	16,600
		11	♂	Barley	34·6	70·8	36·2	3·02	114·81	2·219	16·3	19·3	3·17	11,600
		12	♂	Potato	33·6	59·7	26·1	2·18	116·22	2·231	11·7	13·7	4·45	17,200
		Average barley fed			—	—	31·9	2·7	—	—	16·3	19·2	3·18	11,600
		Average potato fed			—	—	22·6	1·9	—	—	11·6	13·5	4·49	17,400
4	6	19	♂	Barley	29·8	62·0	32·2	2·68	90·30	2·164	14·9	17·4	2·80	10,100
		20	♀	Potato	30·2	52·8	22·6	1·88	92·56	2·189	10·3	11·6	4·10	15,800
		21	♂	Barley	30·9	70·9	40·0	3·33	119·08	2·835	14·1	16·5	2·98	10,700
		22	♂	Potato	31·4	67·6	36·2	3·02	123·32	2·911	12·4	14·0	3·41	13,400
		23	♂	Barley	33·7	68·7	35·0	2·92	110·89	2·648	13·2	15·5	3·17	11,400
		24	♀	Potato	33·3	68·6	35·3	2·94	109·89	2·599	13·6	15·2	3·11	12,100
		Average barley fed			—	—	35·7	3·0	—	—	14·1	16·5	2·98	10,700
		Average potato fed			—	—	31·4	2·6	—	—	12·1	13·6	3·54	13,800
		Average of all barley fed			—	—	29·5	2·5	—	—	15·6	18·7	3·56	12,900
		Average of all potato fed			—	—	23·6	2·0	—	—	12·1	14·3	4·67	18,200

(1) the average weight increase of both barley-fed and potato-fed animals was greater as the level of the casein supplement was raised, although there was little difference in food intake between the subgroups; (2) animals receiving the larger casein supplements made better use of the food energy; though the animals which received the higher levels of casein were less deficient in protein than those which received less casein, their average food intake was not significantly greater. It would therefore seem as if the food consumption of the potato-fed pigs was limited not by the lack of protein but rather

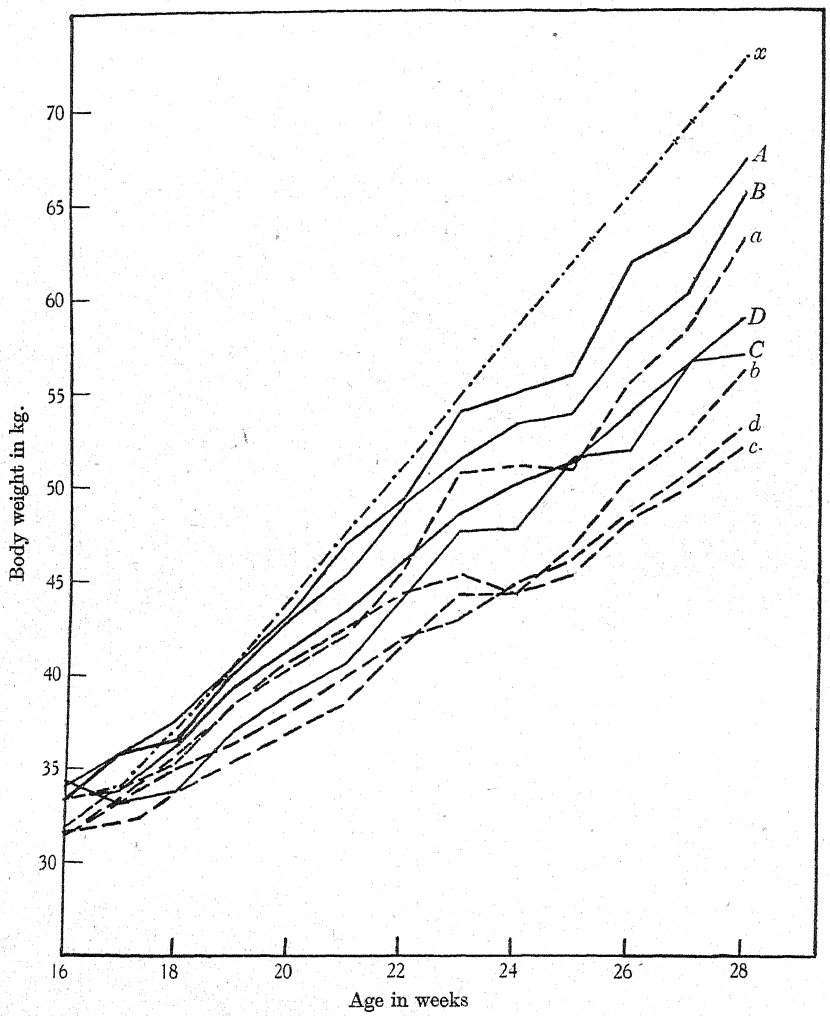

Fig. 1. Average growth curves of pigs in eight subgroups on diets containing barley and potato respectively as staple, with varying proportions of casein. —— signifies barley; ---- signifies potato. Curves *A*, *B*, *C*, *D*: pigs on barley ration + 6, 3, 1% and no casein respectively. Curves *a*, *b*, *c*, *d*: pigs on potato ration + 6, 3, 1% and no casein respectively. Curve *x* = average growth curve for Wessex Saddleback pigs on ordinary commercial pig feed.

(3) the differences in weight increase between barley-fed and potato-fed pigs were roughly similar at all levels of casein intake; (4) the casein therefore supplemented barley N and potato N about equally.

If the food intake of the barley-fed pigs had not been limited to that of the potato-fed pigs, the differences in weight increase between the two groups would have been considerably greater. Although the animals which received the higher levels of casein were less deficient in protein than those which received less casein, their average food intake was not significantly greater. It would therefore seem as if the food consumption of the potato-fed pigs was limited not by the lack of protein but rather by their inability to consume more of their very bulky ration. In other words, this is one of those cases to which Mitchell has frequently drawn attention, in which appetite does not run parallel with the protein efficiency of the ration; and fallacious results would have been obtained had not the food intake of the barley-fed animals been controlled. Since, on the average, the level of casein in the diet

had little effect on food intake, it was not found necessary, as had been anticipated, to equalize the food intakes of the different subgroups.

From Table 5 and Fig. 1 it appears that a supplement of 1 or 3 % of casein to the barley ration gave approximately the same rate of growth increase, and utilization of digestible energy, as a supplement of 6 % casein to the potato ration. In commercial feeding practice, therefore, it would be necessary to feed more than twice as much protein concentrate when potato is substituted for a barley ration of equal N content. Under conditions in which there might be a depression of the digestibility of N consequent on the substitution of potato for barley, it would be necessary to give still more protein concentrate. In practice the growth of the potato-fed pig would be limited by the bulkiness of the ration as well as by its deficiency of N of good biological value.

Section 2. DIGESTIBILITY EXPERIMENTS

METHODS

Six crates were made by placing the individual feeding pens upon wooden tables covered with galvanized iron. Holes were bored through the tables to drain off the urine. The pigs were prevented from moving backwards and forwards by horizontal bars fixed across the back of the pens, and adjusted to fit each pig. The animals soon learnt that the forward end of the crate, where the food appeared, was the more interesting, and there was no trouble from their turning movements in the cage during the collection periods.

After the experiments on growth were concluded, the animals continued to receive the same experimental rations until they were required for the digestibility trials. In all these trials there was a 4-day preliminary period, followed by a 10-day collection period. Food intake was adjusted to the amount which the animals were expected to finish, and was maintained at this level throughout the experiment. There were no refusals of food. The faeces were collected at intervals during the day into buckets placed behind the crates, and the animals conveniently refrained from excreting faeces during the night. The faeces were preserved by moistening with about 2 l. of N H_2SO_4 and analyzed for total N by the Kjeldahl method, and for gross energy in the bomb-calorimeter. Samples of the boiled potatoes were analyzed daily for dry matter and N; the gross energy was determined on a bulk dried sample.

Hogs only were utilized for these experiments, two potato-fed animals and one barley-fed animal being selected from each subgroup. It was necessary to use pig 12, a potato-fed animal, for tests with both 3 and 6 % casein diets, as two of the potato-fed pigs in the subgroup receiving 6 % of casein were gilts; pig 12 had its ration changed between the two tests.

In addition, pigs 11, 12, 17 and 18 were utilized in a further trial (see below) in which approximately half of the ration consisted of boiled potatoes and potato flakes, and approximately half of barley meal. Pigs 11 and 12 also received 6 % casein, while pigs 17 and 18 did not receive any.

RESULTS

Tables 6 and 7 give details of food intakes and faecal excretions, and (apparent) digestibility coefficients. The digestibilities of the N and energy of potato and barley are computed on the assumption that the casein, yeast extract, cod-liver oil, and starch were entirely digested. The digestibility coefficients obtained from tests with different pigs were in close agreement. Pig 8, on the potato ration with 3 % casein, scoured during the experiment, but this did not apparently influence the digestibility coefficients, which were the same as those of its fellow pig 12 on the same ration. In contradistinction to the results of Fingerling [1932], and of Woodman & Evans [1939], the presence of the casein in some of the potato rations did not depress the digestibility either of the N or of the gross energy. The digestibility of the latter was, in fact, high and independent of casein level. The coefficients averaged 93·7 % for energy, and 81·5 % for N, of the potato, and 82·3 % for energy, and 79·1 % for the N, of the barley.

When this experiment was completed, it occurred to us that, although the digestibility of the potato rations had not been lowered by feeding casein in combination with the potato, it might have been depressed if barley had been fed in combination with the potato, either with or without casein; Woodman & Evans [1939] and Fingerling [1932], in the majority of their trials, used barley meal in the basal ration. We therefore undertook a further digestibility trial on pigs 11, 12, 17 and 18. The first batch of fresh 'Majestic' potatoes had by this time deteriorated and, when cooked, usually blackened badly. These potatoes were, however, still palatable, and were given to pigs 11 and 17, while those of the second batch were fed to pigs 12 and 18.

Table 8 gives the results obtained. In computing the digestibility of the potatoes, the casein, yeast extract, cod-liver oil and starch were again assumed to be entirely digested. The mean coefficients obtained for the barley meal in the first series of experiments, viz. 82·3 % for the gross energy and 79·1 % for the N, were taken as the digestibility of the barley meal in the mixed rations. Any associative effect would thus be debited or credited to the boiled potatoes and potato flakes. The average digestibility for the potato energy in the mixed rations was calculated to be 91·0 % with no casein and 92·5 % with 6 % of casein, while that of the N averaged 75·5 and 80·7 % respectively. Thus, addition of casein to the potato ration when barley meal also was present had not depressed the digestibility

Table 6. *'Apparent' digestibility of the nitrogen and energy of potato ration*

Group	Casein in ration (%)	Pig no.	Total dry matter (kg.)	Nitrogen intake (g.) from: Casein	Yeast extract	Potatoes	Total ration	Gross energy intake (kg. cal.) from: Potatoes	Total ration	Nitrogen (g.) in: Faeces	Digested total ration	Digested potato	Gross energy (kg. cal.) in: Faeces	Digested total ration	Digested potato	Digestibility coefficients of: N in total ration	N in potato	Energy in total ration	Energy in potato
1	0	2	15·682	—	4·4	239·0	243·4	57,261	63,363	44·9	198·5	194·1	3550	59,813	53,711	81·6	81·2	94·4	93·8
	0	4	11·955	—	4·4	182·0	186·4	43,625	48,305	28·5	157·9	153·5	2494	45,811	41,141	84·7	84·3	94·8	94·3
2	1	16	9·631	14·2	4·4	147·6	166·2	35,046	39,501	35·3	187·3	161·0	2662	36,389	32,384	79·7	77·1	93·2	92·4
	1	18	14·740	21·9	4·4	196·3	222·6	53,825	59,771	39·7	241·3	174·8	2916	56,855	50,909	84·1	82·0	95·1	94·6
3	3	8	13·994	62·1	4·4	214·5	281·0	50,917	57,140	38·9	227·7	157·4	3280	53,860	47,637	85·9	81·5	94·3	93·6
	3	12	14·740	65·9	4·4	196·3	266·6	53,825	60,192	41·8	343·5	199·5	3454	56,738	50,371	85·4	80·2	94·3	93·6
4	6	12	15·749	139·6	4·4	241·3	385·3	57,330	64,976	39·7	324·5	188·3	3860	61,116	53,470	89·2	82·7	94·1	93·3
	6	22	14·874	131·8	4·4	228·0	364·2	54,126	61,366	39·7	324·5	188·3	3447	57,919	50,679	89·1	82·6	94·4	93·6
															Average	85·0	81·5	94·3	93·7

Table 7. *'Apparent' digestibility of the nitrogen and energy of barley-meal ration*

Casein in diet (%)	Pig no.	Food intake (kg. dry matter)	Nitrogen intake (g.) in: Barley meal	Casein	Yeast	Total ration	Gross energy intake: As barley (kg. cal.)	Total ration (kg. cal.)	Nitrogen (g.) in: Faeces	Digested total ration	Digested barley	Gross energy (kg. cal.) in: Faeces	Digested total ration	Digested barley	Digestibility coefficients of: N in total ration	N in barley	Energy in total ration	Energy in barley
0	3	12·374	184·9	—	4·4	189·3	41,453	51,835	35·4	153·9	149·5	6,471	45,364	34,982	81·3	80·9	87·5	84·4
1	17	15·947	238·2	24·1	4·4	266·7	53,422	67,041	49·2	217·5	189·0	9,822	57,219	43,600	81·6	79·3	85·3	81·6
3	11	15·947	238·2	72·7	4·4	315·2	53,422	67,504	48·2	267·0	196·0	9,029	58,475	44,393	84·7	79·8	86·6	83·1
6	21	15·085	225·4	137·1	4·4	366·9	50,535	64,519	53·6	313·3	171·8	10,138	54,381	40,397	85·4	76·2	84·3	79·9
														Average	83·2	79·1	85·9	82·3

Table 8. *'Apparent' digestibility of the nitrogen and energy of potatoes when fed in a mixed diet consisting of potatoes, barley meal and casein**

Group	Pig no.	Food intake (kg. dry matter): Potatoes	Barley ration	Total ration	Nitrogen intake (g.) in: Barley	Casein	Yeast	Gross energy intake (kg. cal.) in: Potatoes	Total ration	Barley	Total ration	Nitrogen (g.) in: Faeces	Digested total ration	Faeces due to potatoes	Digested potato	Gross energy (kg. cal.) in: Faeces due to potatoes	Total faeces	Digestibility coefficients of: N in total ration	N in potato	Energy in total ration	Energy in potato
0 % casein	17	8·338	7·974	16·312	119·1	—	4·4	123·9	247·4	30,657	26,713	67,224	56·9	190·5	31·9	92·0	3143	8141	77·0	74·3	87·9	89·8
	18	7·792	7·974	15·766	119·1	—	4·4	120·0	243·5	28,322	26,713	64,888	49·6	193·9	24·6	95·4	2246	6974	79·6	76·7	89·3	92·1
6 % casein	11	8·338	7·974	16·312	119·1	142·3	4·4	123·9	389·7	30,657	26,713	68,593	51·5	338·2	26·5	97·4	2482	7210	86·7	78·6	89·6	91·9
	12	7·792	7·974	15·766	119·1	142·3	4·4	120·0	385·0	28,322	26,713	66,257	46·2	339·6	20·7	99·3	1977	6725	88·0	82·8	89·9	93·0

* The digestibility of potato is calculated by assuming both energy and N of casein and yeast to be 100 % digestible, energy of barley meal to be 82·3 % digestible and N of barley meal to be 79·1 % digestible. The potatoes fed to pigs 11 and 17 were from batch 1 and those fed to pigs 12 and 18 from batch 2.

of either energy or N. We had not expected that there would be any effect on the digestibility of the energy, but had anticipated from the results obtained by Fingerling and by Woodman & Evans that the digestibility of the N might be lowered. The average digestibility coefficients of the potato in the rations containing the second batch of 'Majestic' potatoes (pigs 12 and 18) were 92·6 % for energy and 80·1 % for N, compared with average values of 93·7 and 81·5 % obtained with the same potatoes in the first series of trials (Table 6). The presence of the barley with the potatoes, therefore, caused no lowering of the digestibility of the ration. In short, these three feeding stuffs, potatoes, barley, and casein, gave the same digestibility coefficients when fed in a mixture as they gave, or in the case of casein, were assumed to give, when fed separately.

Our experiments, therefore, do not shed any light on the perplexingly low digestibility coefficients of potato N encountered by other workers. Both Fingerling and Woodman & Evans included fish meal in their basal rations and had we also used this concentrate in place of casein our results might possibly have been different. It is more probable, however, that differences between different samples of potatoes or methods of processing were responsible for the discrepancy. The digestibility of potatoes in the rations containing boiled potatoes from first batch of 'Majestics' averaged 90·9 % for energy and 76·9 % for N (pigs 11 and 17, Table 8); that of the 'Majestic' potatoes of the second batch averaged 93·7 % for energy and 81·5 % for nitrogen (see Table 6). There was, therefore, little difference in digestibility between the two samples of 'Majestic' potatoes which we used. Since the figures for potato digestibility given in Table 6 were obtained from experiments in which there were more animals, and in which there was no admixture with barley meal, they are considered to be the more accurate, and these figures only have been employed in the computation of the digestible nutrients in the growth experiments described above.

SUMMARY

1. The value of the nitrogen of the potato was compared with that of barley for supporting growth of 'fattening pigs'. The observations were made upon 12 pairs of similar animals, one of each pair receiving a potato, and the other a barley, diet.

2. The rations compared contained equal amounts of nitrogen derived from potato or barley, with and without supplementary nitrogen from casein. Half of the potato ration was composed of fresh boiled (Majestic) potatoes and half of commercial dried potato flakes. The total nitrogen of the diets varied from 1·5 % when no casein was given, to 2·4 % when 6 % casein was included, the figures being calculated on the dry weight.

3. The food intake, on dry weight basis, of each barley-fed pig was limited to the amount consumed by its potato-fed partner.

4. The value of the potato nitrogen, whether fed alone or supplemented with that of casein, was consistently inferior to that of the barley nitrogen. The kg. weight increase per kg. digestible nitrogen intake varied from 16·5 to 20·3 for barley rations and from 13·5 to 15·2 for corresponding potato rations. The kg. (dry) food ingested, per kg. weight increase, varied from 3·0 to 4·1 for the barley, and from 3·5 to 5·6 for the potato, rations. The conversion of food into live weight was thus more economical in the case of barley.

5. Addition of casein increased the economy in the conversion of food into live weight to about the same slight extent when either potatoes or barley were fed. The conclusion drawn is that casein nitrogen supplements equally that of potatoes and barley.

6. Some evidence was obtained that the potato-fed pigs were fatter for their carcass weight than the barley-fed pigs. Those which received no casein or only 1 % in their diet were also relatively fatter for their body weight than those receiving 3 or 6 % (see Appendix 1).

7. The average values for the coefficient of (apparent) digestibility of a mixture in equal parts of potato flakes and fresh boiled potatoes was 93·7 % for energy and 81·5 % for total nitrogen. Corresponding figures for barley were 82·3 and 79·1 %.

8. The digestibility of potatoes was unaffected by admixture with casein up to 6 % of the diet, assuming the latter to be entirely digested. It was also unaffected by admixture with barley to about 50 % of the diet, assuming that the coefficient of digestibility of the barley was the same as that ascertained when the barley was fed alone or with casein.

9. The rate of increase of weight when 3 % casein was added to the barley ration (the total nitrogen being 1·95 %) was about the same as when 6 % was added to the potato ration (the total nitrogen being 2·4 %). The intake of food was not very different in the two groups.

In conclusion we wish to thank Sir Charles Martin not only for his hospitality at Roebuck House, Chesterton, but also for constant help and advice throughout the work, Professor Dalling for the facilities given at the Institute of Animal Pathology where the pigs were kept, and Dr Harriette Chick for her help in preparation of this paper. Dr E. H. Callow, of the Low Temperature Research Station, Cambridge, gave us valuable assistance in the interpretation of the carcass data.

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Appendix 1. Examination of Carcasses. (By J. C. D. HUTCHINSON and A. N. WORDEN)

As described in the main portion of the text, not all the pigs were killed at the termination of the growth experiments, the majority being required for the subsequent digestibility trials. During the first of the two digestibility trials, pig 12 received a diet containing 6 % casein instead of 3 % as previously, while during the second trials pigs 11, 12, 17 and 18 received mixed rations of barley and potato with or without casein. The food intake (on dry matter basis) of the pigs fed, respectively, on potato and barley rations continued to be paired, however, and the animals were killed off in pairs as soon as each digestibility trial was concluded.

The carcass measurements are recorded in Table 9. The carcass included the head, feet, flare fat and kidneys, and was weighed within 30 min.

Table 9. *Carcass measurements*

Pig no.	% casein in diet	Warm carcass weight (lb.)	Length of body cavity (mm.)	Back-fat (mm.) A	B	C_3	C_2	C_1	Calculated value of *T* (mm.)	*B* − *T* (mm.)
1 ♂ B	0	138	822	54	27	42	39	43	21·5	5·5
2 ♂ P		129	849	51	26	31	30	37	20·2	5·8
3 ♂ B		109	800	49	20	35	29	34	17·2	2·8
4 ♂ P		96	760	43	20	32	31	33	15·3	4·7
5 ♀ B		87	646	38	13	22	15	18	14·0	(−1·0)
6 ♂ P		75	612	43	19	29	27	25	12·2	6·8
	Average: Barley-fed		756	—	20·0	—	—	—	17·6	2·4
	Potato-fed		740	—	21·6	—	—	—	15·8	5·8
13 ♂ B	1	113	713	44	18	25	21	26	17·8	0·2
14 ♀ P		99	652	45	18	24	20	23	15·8	2·2
15 ♀ B		86	760	34	15	24	21	23	13·8	1·2
16 ♂ P		81	746	45	17	28	24	31	13·1	3·9
17 ♂ B		154	874	63	29	41	41	47	23·8	4·2
18 ♂ P		147	837	54	29	40	43	39	22·7	6·3
	Average: Barley-fed		782	—	20·6	—	—	—	18·4	2·2
	Potato-fed		745	—	21·3	—	—	—	17·2	4·1
7 ♀ B	3	139	845	45	19	27	21	28	21·7	(−2·7)
8 ♂ P		112	790	42	23	34	26	39	17·5	5·5
9 ♂ B		108	732	40	16	26	19	30	17·1	(−1·1)
10 ♀ P		96	666	37	18	26	20	26	15·3	2·7
11 ♂ B		159	886	59	26	41	30	36	24·5	1·5
12 ♂ P		148	873	51	25	35	33	38	23·0	2·0
	Average: Barley-fed		821	—	20·3	—	—	—	21·1	(−0·8)
	Potato-fed		776	—	22·0	—	—	—	18·6	3·4
19 ♂ B	6	115	806	41	17	26	25	31	18·1	(−1·1)
20 ♀ P		105	791	40	20	32	27	35	16·7	3·3
21 ♂ B		146	845	44	23	33	32	39	22·6	0·4
22 ♂ P		144	852	52	21	33	27	39	22·3	(−1·3)
23 ♂ B		123	711	41	21	26	20	27	19·3	1·7
24 ♀ P		120	734	41	19	27	22	29	18·9	0·1
	Average: Barley-fed		787	—	20·3	—	—	—	20·0	0·3
	Potato-fed		792	—	20·0	—	—	—	19·3	0·7

of the death of the animal. The cold carcass weight was obtained by suitable subtraction from the warm carcass weight, i.e. 3 lb. from weights up to 143 lb., and 4 lb. from weights of 144–189 lb. The subcutaneous fat was measured at (A) the shoulder, (B) the last rib, and (C_3, C_2, C_1) the front, middle and back, respectively, of the rump muscle. The length of the body cavity was measured (on the warm carcass suspended from a gambrel) from the pubic symphysis to the junction of the sternum with the first rib [Davidson, Hammond, Swain & Wright, 1937]. The value T was calculated from the equation, $T = 1{\cdot}4 + 0{\cdot}149W$, where W is the cold carcass weight [Callow, 1942]; T represents the expected thickness of the back fat at the thinnest portion, i.e. over the last rib. The difference between the observed and calculated values ($B - T$) for this measurement are given in the last column of Table 9, and are discussed in Appendix 2.

In most cases our animals were fatter than expected, i.e. than average pigs of the same carcass weight. Callow's formula, derived from a large number of pigs of various breeds, may possibly not fit our particular Wessex Saddlebacks. Nevertheless, it is clear that the potato-fed pigs were relatively fatter, in relation to their carcass weight, than their barley-fed partners, and that the pigs which received no casein or 1 % were relatively fatter than those which received 3 or 6 % casein.

While it is not possible to draw definite conclusions, in view of the possible inapplicability of Callow's formula, the figures suggest that deficiency of nitrogen may affect the composition of the carcass. Those animals which received the most nitrogen of high biological value were relatively the leanest in relation to their carcass weight, thus indicating extra muscle deposition.

McMeekan [1940] could not detect any influence of the level of dietary protein on the body composition of the growing pig. The low protein diet which he used, however, supported optimal growth, and the food intakes of his animals were not limited as in our experiment. Forbes, Swift, Black & Kahlenberg [1935] and Forbes, Voris, Bratzler & Wainio [1938], working with rats whose food intake was paired, found that the nitrogen/fat ratio of the body gains increased as the dietary protein level was raised, and Hamilton [1939] arrived at the same conclusions from a similar experiment.

Appendix 2. Statistical Analysis. (By J. O. Irwin, Medical Research Council's Statistical Staff, and J. C. D. Hutchinson)

Table 10 gives the analysis of variance of (*a*) body weight increase in kg./kg. of nitrogen intake, (*b*) body weight increase in kg./kg. of digestible nitrogen intake, (*c*) dry food intake in kg., (*d*) digestible energy in kg. cal./kg. increase in body weight, and (*e*) the difference between the thickness of back fat at the last rib and the average figure for a carcass of the same weight ($B - T$ in Table 9).

In each casein subgroup every pig on a barley ration was paired with another, on a potato ration, of live weight as nearly as possible the same, and their food intakes were similarly paired. Thus the direct effect of casein level is evaluated by an analysis of variance within and between casein groups of the *sums* for two pigs. The difference between the effects of the potato and barley rations, on the other hand, and the effect of addition of casein on this difference—that is, the interaction between the main and subsidiary comparisons—were evaluated by an analysis of variance of the *differences* between the pairs of pigs. In consequence of the pairing, the error for the direct casein comparison is larger than that for the other two comparisons.

In every case there is a significant difference between the two main groups fed respectively on potatoes and barley. The direct effect of the casein additions is not significant in any of the groups but approaches significance for food intake and for digestible energy intake per kg. weight increase and for thickness of back fat. All three values are on the average less at the higher levels of casein. This fact, which agrees with the result of the previous experiment of Macrae *et al.* [1942], suggests that this non-significance is due to the relatively large error of experiment and that the effect could be shown to be real in an experiment conducted with a larger number of animals.

Table 10. *Analyses of variance*

Variation due to	Degrees of freedom	Weight increase (kg./kg. N intake) Sum of squares	Mean sq.	Variance ratio	Weight increase (kg./kg. digestible N intake) Sum of squares	Mean sq.	Variance ratio	Food intake (kg./kg. wt. increase) Sum of squares	Mean sq.	Variance ratio	Intake of digestible energy (kg. cal./kg. wt. increase) Sum of squares	Mean sq.	Variance ratio	Back fat. Difference between thickness of back fat at last rib and that calculated from warm carcass wt.† Sum of squares	Mean sq.	Variance ratio
Casein level	3	5·81	1·94	0·31	21·95	7·32	0·79	9·263	3·088	3·67	139·36	46·45	3·92	49·18	16·39	3·87
Error for casein	8	50·34	6·29	—	73·85	9·23	—	6·727	0·841	—	94·81	11·85	—	33·85	4·23	—
Potato compared with barley	1	73·50	73·50	*46·52**	119·71	119·71	*57·00*	7·404	7·404	*24·17*	164·33	64·33	*37·52*	36·75	36·75	*6·92*
Interaction	3	6·73	2·24	1·42	8·39	2·80	1·33	0·800	0·267	0·87	13·54	4·51	1·03	12·78	4·26	0·80
Error	8	12·60	1·58	—	16·83	2·10	—	2·450	0·306	—	35·02	4·38	—	42·50	5·31	—
Total	23	148·98			240·73			26·644			447·06			175·06		

* Values of the variance ratios in italics denote a significant effect.

† $(B - T)$ in Table 9.

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Respiration and Nutritional Requirements of Certain Members of the Genus *Mycobacterium*

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Much investigation [reviewed by Calmette, 1923; Long, 1923; Knight, 1936] has shown that many acid-fast bacilli can be cultivated in synthetic media. The utilization of specific carbon and nitrogen sources has also been studied [Kendall, Day & Walker, 1914; Long, 1921–22; see also Knight, 1936; Merrill, 1931].

With the exception of Uyei [1927], who employed the tubercle bacillus, authors have not defined precisely the size of the inoculum, and this is now known to be a critical factor in experiments on bacterial nutrition. Accordingly, we have reinvestigated the growth requirements of certain *Mycobacteria* in order to determine the effects of varying the size of inoculum. The use of defined inocula and other rigid conditions has made it possible to demonstrate the stimulant action of certain accessory nutrients in an unequivocal manner.

EXPERIMENTAL

Organisms. The following species were utilized: *Myco. phlei* a (Veterinary Research Station, Glenfield, N.S.W.), *Myco. phlei* b (McMaster Veterinary Laboratory, Sydney), *Myco. phlei* (no. 525), *Myco. smegmatis* (no. 523), *Myco. stercoris* (no. 3820), *Myco.* sp. *Karlinski* (no. 2071), *Myco.* sp. *leprous Kedrowsky* (no. 509), *Myco. ranae* (no. 2891), *Myco. butyricum* (no. 337), *Myco. tuberculosis hominis*, avirulent strain T_1 (Commonwealth Serum Laboratories, Melbourne; originally from the Royal Institute of Public Health, London), *Myco. tuberculosis hominis*, virulent strain H 37 (Commonwealth Serum Laboratories, Melbourne; originally from the U.S.A.). The species designated by catalogue numbers were obtained from the National Collection of Type Cultures, Lister Institute, London.

In this laboratory, stock cultures were maintained on Dorset's egg medium and subcultured every fifth week. Prior to experimental use the organisms were grown for several generations on the surface of meat infusion broth containing 5% of glycerol. Broth cultures, except those of tubercle bacilli, were 7 days old when used. All organisms were cultivated at 38°. The cultural characteristics of the three specimens of *Myco. phlei* were identical.

METHODS

Washed bacterial suspensions. In both respiratory and nutritional experiments the bacteria were removed from the surface of the broth, washed three times with sterile distilled water (by centrifugation), and a known weight resuspended in a measured volume of water. The notorious difficulty of obtaining homogeneous suspensions of acid-fast bacilli was overcome in two ways as follows:

(*a*) *Preparation of inocula for nutritional tests.* Small quantities of bacteria (up to 60 mg. wet wt.) were successfully dispersed by light hand-grinding with 0·5 ml. of water in a test-tube and plunger apparatus made from two closely fitting tubes, the contiguous surfaces of which had been carefully ground. The suspension was washed from the grinder and diluted with sterile distilled water.

(*b*) *Preparation of suspensions for manometric work.* Larger quantities (up to 2 g. wet wt.) were dispersed by gentle shaking in a small stainless steel ball-mill similar to that described by Langner & Forrester [1939]. The mill, containing bacteria, 10 ml. of sterile distilled water and three steel balls (diam. $\frac{3}{8}$ in.), was clamped to a Warburg shaker and shaken outside the thermostat for 20 min. at the rate of 40 oscillations/min. The suspension was diluted to an appropriate volume. Microscopic examination of stained smears revealed no disruption of bacterial substance or loss of acid-fast properties.

Bacterial suspensions prepared by these methods are satisfactory for the work described, and possess a degree of stability approximating to that of a yeast suspension of corresponding density. Suspensions of *Myco. smegmatis* and *Myco. stercoris* tend to flocculate more rapidly than the others.

Measurement of respiration. Oxygen consumption was measured by the manometric method of Warburg, the usual conical vessels being employed. The gas space contained oxygen and the temperature of the thermostat was 38°. The main compartment of the Warburg cup contained 1 ml. of 0·2 *M* sodium phosphate buffer, pH 7·4, to which were added 2 ml. of bacterial suspension. Substrate solutions were added from the side-bulb after 30 min. equilibration. Experiments were conducted for times ranging from 1 to 4 hr.

Oxygen uptake (Q_{O_2}) is expressed as μl. O_2 consumed/mg. dry wt. bacteria/hr. The dry weight was determined by drying 6 ml. of suspension to constant weight at 107°. 2 ml. of suspension generally contained 12–15 mg. dry weight of bacteria.

Nutritional experiments. In this work we have adhered as closely as possible to the principles stressed by Knight & Fildes [1933] and Fildes & Richardson [1935]. In adapting these principles to experiments on the slowly growing *Mycobacteria* the chief considerations were the use of a fluid synthetic medium containing substances of the highest purity, and the addition of a washed inoculum which was

large enough to produce growth in a reasonable time, but not so heavy as to carry over significant amounts of medium from the mother culture or to provide significant quantities of growth stimulants arising from cellular disintegration.

In order to ascertain the requisite inoculum and to test the ability of each organism to grow with different sources of carbon, exploratory experiments were conducted with $6 \times \frac{3}{4}$ in. test-tubes containing 5 ml. of medium. The inoculum used initially was 1 mg. wet weight of bacilli in one drop (0·05 ml.) of suspension; the result was a guide to the inoculum permissible in decisive experiments, all of which were performed in 20 ml. of medium contained in 100 ml. pyrex Erlenmeyer flasks. The depth of fluid was 6–8 mm.

A series of parallel experiments, in which two lots of bacteria were washed from 3 to 9 times, one with distilled water and the other with 0·9 % NaCl, gave identical results, showing that three washings were adequate, and that water was no more deleterious to the bacteria than saline.

In all experiments the ammonium ion was either the sole or the chief source of nitrogen. Organic materials were synthetic products, or analytical reagents where these were available; inorganic materials were of analytical grade. The following solutions were prepared:

(*a*) Basal salt mixture contained 1·0 g. of $(NH_4)_2SO_4$; 4·5 g. of KH_2PO_4; 2·0 g. of NaCl; 0·25 g. of $NaHCO_3$; 26·0 ml. of NaOH and distilled water to make the total volume 600 ml.

The pH was 7·5. The solution was distributed in test-tubes (3 ml.) or in Erlenmeyer flasks (12 ml.) and autoclaved at 15 lb. for 15 min.

(*b*) 0·2 % $MgSO_4$, $7H_2O$ (autoclaved).

(*c*) 10 % solutions of various carbon-containing substances (carbon-source) sterilized by filtration (Seitz EK) or by autoclaving, according to heat stability. Acids were used as neutral sodium salts.

(*d*) Solutions of substances under test, which were added in small volume and in low concentrations, were filtered or autoclaved according to their stability. Whenever stability was in doubt, both autoclaved and filtered specimens were tested. Acids were neutralized with NaOH, bases with HCl.

Solutions of classes (*b*), (*c*) and (*d*) were added aseptically to the distributed salt mixture, and, after all additions had been made, sterile distilled water was added to bring the final volume to 5 ml. in test-tubes, and to 20 ml. in flasks. Thus 600 ml. of original salt mixture were diluted to 1000 ml. The final concentration of magnesium sulphate was 0·04 g./litre, and that of carbon source 1–2 %.

Media were inoculated by cautious addition of 0·05 ml. of bacterial suspension to the surface, which was subsequently disturbed as little as possible. Test-tubes were slanted on trays. All vessels were plugged with cotton-wool, remaining unsealed unless experiments were prolonged beyond 7 days. Cultures were incubated at 38° and observed daily.

At the termination of an experiment the growth was transferred to tightly packed asbestos on a Gooch crucible, washed three times with distilled water, and dried to constant weight at 107°. Although this procedure involves a small loss of bacteria, the weights are sufficiently accurate for the purpose of the experiments described.

RESULTS

Respiration measurements

The oxygen consumption of several species in the presence and absence of substrates is shown in Table 1. When no substrate is added, there is an oxygen uptake which remains constant for about 2 hr. and diminishes slowly thereafter. The value of Q_{O_2} varies with the species, and bears some relationship to the rate of growth in nutrient broth. In the presence of most substrates oxygen consumption increases above the 'resting' value. There is one notable exception: citrate causes no significant change in respiration under the conditions used. Moreover, there was no oxidation of citrate when the suspension medium was phosphate saline [Krebs, 1933] or the basal salt mixture described above. Bacterial suspensions which have been heated in a boiling water-bath for 10 min. have no oxygen uptake either in presence or absence of added substrate.

Table 1. *Respiration of* Mycobacteria *with various substrates*

Substrate (0·02 *M*)	Uptake of oxygen (Q_{O_2}) with *Myco. phlei*	*Myco. smegmatis*	*Myco. stercoris*	*Myco.* sp. *Karlinski*	*Myco. ranae*	*Myco.* sp. *leprous Kedrowsky*	*Myco. butyricum*	*Myco. tuberculosis hominis* T 1
None	− 7·1	−16·2	−12·1	−18·9	−11·4	− 7·7	− 9·2	−2·0
Glucose	−27·9	−22·6	−15·3	−21·9	−32·3	− 7·9	−12·9	−3·8
Fructose	− 9·9	−26·6	−15·5	−26·3	−20·1	− 8·5	−15·5	—
Glycerol	− 8·8	−27·7	−23·0	−35·2	−20·5	−10·4	−27·1	−3·2
Sodium *dl*-lactate	−25·7	−42·6	−27·5	−36·2	−16·1	− 9·1	−11·0	−3·7
Sodium pyruvate	−11·3	−31·9	−24·2	−34·9	−35·9	− 9·8	−15·4	−2·6
Sodium acetate	− 9·3	−40·1	−25·2	−46·9	−30·6	−13·3	−22·3	−2·5
Sodium succinate	−11·2	−26·6	−22·3	−24·7	−18·1	− 8·0	− 9·3	—
Sodium citrate	− 7·1	−15·2	−11·9	−19·9	−12·6	− 7·5	− 9·1	−2·3
dl-Alanine	− 8·7	−17·4	−14·1	−22·6	−20·8	− 8·1	−10·3	—

(Medium: 0·066 *M* phosphate buffer, pH 7·4, at 38°. Gas: oxygen.)

It will be seen that the members of the acid-fast group exhibit considerable differences in their ability to oxidize the same substrate. For example, the respiration rate of *Myco. phlei* is increased three- to fourfold by glucose and lactate, whereas pyruvate, acetate, succinate and glycerol are oxidized slowly; on the other hand, the oxygen consumption of *Myco. ranae* is rapid in presence of pyruvate, acetate and glucose, but much slower in presence of lactate and succinate. *Myco. butyricum* shows preference for glycerol and acetate, whilst *Myco.* sp. *Karlinski* presents the highest rate with acetate, and distinctly lower—though still considerable—rates with glycerol, lactate and pyruvate.

Growth in synthetic media

Utilizable carbon sources

If the metabolism of the *Mycobacteria* is predominantly aerobic—as generally believed—there should be some relationship between the rate of oxidation of a given substrate and rate of growth in a synthetic medium containing that substrate as the sole source of carbon. In the experiments described in Table 2, the concentration of carbon source was 1 % and the inoculum was relatively heavy (1 mg. wet wt. bacilli/tube). Gross differences in rate of growth were assessed by daily inspection. Although the first visible evidence of multiplication was often the appearance of 'bottom granules', the chief mass of growth was confined to the surface.

The results demonstrate that certain species of *Mycobacteria* are able to grow when heavily seeded in synthetic media containing carbonaceous material which they can oxidize: and that, in general, the rate of growth shows some degree of correspondence to the increased oxygen uptake in phosphate buffer (Tables 1, 2). With citrate there was no increased oxygen consumption, and there was no growth in synthetic medium.

Selection of an inoculum suitable for critical experiments

Bacterial suspensions were prepared, by serial dilution, to contain amounts of wet bacilli varying from 10^{-1} to 10^{-8} mg./drop (0·05 ml.). Although bacilli, both clumped and isolated, could be detected microscopically, turbidity was no longer visible in a suspension of 10^{-4} mg./drop. Several tubes of glycerol broth were inoculated with each dilution, 1 drop to 5 ml. of medium. Subsequent growth indicated that suspensions containing less than 10^{-5} mg./0·05 ml. have a random distribution of bacilli, which renders them unsuitable for nutritional experiments [cf. Berg, 1941]. Suspension of 10^{-1} to 10^{-5} mg./drop gave growth in all tubes. With 10^{-1} to 10^{-3} mg./drop, growth appeared on the third day, but with 10^{-4} mg./drop not until the fifth day.

Hence an inoculum of 10^{-2} mg. was selected for most of the work, because it ensured approximately equal seedings and growth in a reasonable time. This inoculum contained about 500,000 bacilli. *Myco. ranae* is exceptional in that it shows less tendency to clump, and so it may be used in higher dilutions.

Growth with small inocula

The results of seeding 20 ml. of synthetic medium, and of glycerinated broth, with relatively small quantities of bacteria are shown in Table 3. When no growth occurred in 7 days, the plug was sealed with paraffin wax and incubation continued.

The ability of an inoculum of 0·01 mg. to multiply to considerable proportions in 7 days is illustrated by the figures for glycerol broth. In synthetic media, however, there is no growth if the carbon source is glucose, fructose, glycerol, acetate, succinate or citrate. Lactate and pyruvate are exceptional. *Myco. phlei*, *Myco. smegmatis* and *Myco. Karlinski* grew in both lactate and pyruvate, whilst *Myco. stercoris* produced slight growth in lactate.

Table 3 also shows that an inoculum of 0·01 mg. remains viable after 7 days, and is able to attain weighable proportions with certain sources of carbon. *Myco. smegmatis* reached a growth of 7·5 mg. in succinate after 5 weeks; *Myco. Karlinski*, 15 mg.

Table 2. *Growth of* Mycobacteria *in synthetic media containing various sources of carbon*

Organism	Growth with source of carbon as 1 % of: Glucose	Fructose	Glycerol	*dl*-Lactate	Pyruvate	Acetate	Succinate
Myco. phlei	+ + +	±	±	+ + + +	+ + +	+ +	+
Myco. smegmatis	+ + +	+ + + +	+ + + +	+ + +	+ + + +	+	+
Myco. stercoris	+ +	+ + +	+ + + +	+ + + +	+ + + +	+	±
Myco. sp. *Karlinski*	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +
Myco. ranae	+ +	+ + + +	+	±	+ + + +	+ + + +	+ +
Myco. sp. *Kedrowsky*	±	+ +	+ +	±	+ + + +	+ + +	+
Myco. butyricum	+	±	+	±	+	±	±

With citrate (0·5 %) as source of carbon, no growth occurred with any organism.
Number of positive signs indicates rate of growth. ± = trace of growth.
(Nitrogen source, $(NH_4)_2SO_4$. Time, 7 days. Inoculum, 1 mg. seeded in 5 ml. of medium.)

Table 3. *Growth of* Mycobacteria *in synthetic media in presence and absence of citrate*

Organism	Duration (days)	Inoculum (mg.)	Growth (mg. dry wt.) with source of carbon as 1% of: Glucose Control	Glucose Citrate	Fructose Control	Fructose Citrate	Glycerol Control	Glycerol Citrate	*dl*-Lactate Control	*dl*-Lactate Citrate	Pyruvate Control	Pyruvate Citrate	Acetate Control	Acetate Citrate	Succinate Control	Succinate Citrate	Control glycerol broth
Myco. phlei	7	0·01	Nil	10·1	Nil	Trace	Nil	Nil	15·2	24·2	15·0	20·1	Nil	9·0	Nil	Trace	46·0
		0·001	—	—	—	—	—	—	14·1	22·0	—	—	—	—	—	—	—
		0·0001	—	—	—	—	—	—	2·0	20·7	—	—	—	—	—	—	—
	21	0·01	10·2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	42	1·0	—	—	—	—	Trace	14·2	—	—	—	—	—	—	—	—	—
	56	0·01	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1·0	—	—	—	—	8·4	—	—	—	—	—	—	—	—	—	—
Myco. smegmatis	7	0·01	Nil	14·0	Nil	23·4	Nil	17·0	42·2	44·8	13·3	27·2	Nil	9·8	Nil	16·3	50·2
	35	0·01	—	—	Nil	—	—	—	—	—	—	—	Nil	—	7·5	—	—
	63	0·01	—	—	Nil	—	—	—	—	—	—	—	Nil	—	—	—	—
Myco. stercoris	7	0·01	Nil	13·7	Nil	20·8	Nil	6·2	Trace	28·9	Nil	21·2	Nil	15·3	Nil	14·7	37·1
	21	0·01	—	—	Nil	—	Nil	—	27·8	—	Nil	—	Nil	—	Nil	—	—
	63	0·01	—	—	Nil	—	Nil	—	—	—	Nil	—	Nil	—	Nil	—	—
Myco. sp. *Karlinski*	7	0·01	Nil	8·7	Nil	9·1	Nil	6·0	3·1	18·9	3·7	16·2	Nil	2·0	Nil	4·4	33·4
		0·001	Nil	7·4	—	—	—	—	11·3	27·7	Trace	13·2	—	—	—	—	—
		0·0001	Nil	4·5	—	—	—	—	—	—	Nil	24·7	—	—	—	—	—
	42	0·01	—	—	Nil	—	Nil	—	—	—	—	—	15·0	—	Nil	—	—
	63	0·01	—	—	Nil	—	Nil	—	—	—	—	—	—	—	Nil	—	—
Myco. sp. *Kedrowsky*	7	0·01	Nil	Trace	Nil	7·0	Nil	2·5	Nil	—	3·9	5·3	Nil	3·5	Nil	—	5·8
	35	0·01	Nil	—	Nil	—	Nil	—	Nil	—	—	—	Nil	—	4·5	—	—
Myco. butyricum	7	0·01	Nil	Nil	Nil	Nil	Nil	Trace	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	22·5
	21	0·01	Nil	Nil	Nil	Nil	Nil	—	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	—
Myco. ranae	7	0·0001	Trace	5·1	Nil	Nil	Trace	0·1	Nil	10·1	1·8	9·7	Trace	2·3	Nil	Nil	6·6
	14	0·0001	—	—	Trace	8·2	—	—	—	—	—	—	—	—	Nil	Nil	—

(Nitrogen source, $(NH_4)_2SO_4$. Volume of medium, 20 ml. Conc. of sodium citrate, 0·01%.)

after 6 weeks in acetate. *Myco. stercoris*, which showed only a trace of growth in lactate after 1 week, increased to 27·8 mg. in 3 weeks, and *Myco. Kedrowsky* presented a weighable growth in pyruvate after 5 weeks, although no trace was visible at the end of the first week. *Myco. phlei* subsisting on glucose gave somewhat irregular results: in some experiments growth began after 10 days and increased to 10 mg. by the end of the third week, whereas in others no growth was obtained in 9 weeks. None of the organisms studied showed any growth in synthetic medium containing citrate as the sole source of carbon, even when the flasks were incubated for 20 weeks.

Growth usually began as small 'bottom granules' and later extended to cover the surface with a thin film, the thickness of which varied with the organism. In lactate, growth was always confined to the surface, where it began as a thin, veil-like pellicle. *Myco. phlei* presented its normal pigmentation only in the presence of glycerol [see Ingraham & Steenbock, 1935]; with other sources of carbon the pigmentation was faint. *Myco. ranae*, which is emulsified with ease, grew so rapidly at 38° that the inoculum was reduced to 0·0001 mg.

The experiments recorded in Table 3 show that the amount of growth in synthetic media is a function of both time and size of inoculum.

The stimulant effect on growth of the addition of various substances to the medium

The effect of citrate. The effect of adding a small quantity of citrate can be seen in Table 3. Uniform results, quantitatively of the same order, were obtained with five different samples of commercial citric acid, and also with a specimen of synthetic citric acid prepared by the method of Dünschmann [1891]. Controls containing citrate as the sole carbonaceous material in concentrations ranging from 0·0001 to 2·0 % have never shown the slightest trace of growth.

Table 3 shows that when the size of the inoculum and the time of the experiment are fixed arbitrarily, a small amount of citrate (0·01 %) can be a factor which determines growth in a synthetic medium; and that when some growth occurs in the absence of citrate, it always reaches greater proportions in the presence of citrate. The yields were unaffected by doubling the usual concentrations of the sources of carbon and nitrogen. The effect occurs with all organisms except tubercle bacilli (see later) and *Myco. butyricum*, which begins to grow in the presence of glycerol and citrate, but ceases after a short time.

The citrate effect was examined more extensively, *Myco. phlei* being used as a convenient organism. Table 4 illustrates the influence of concentration.

Table 4. *Growth of* Myco. phlei *in a synthetic medium containing citrate*

Incubation (days)	Inoculum (mg. wet wt.)	Conc. of citrate (%)	Bacterial growth (mg. dry wt.)
7	0·01	Nil	Nil
		0·1	7·7
		0·01	8·1
		0·001	Nil
		0·0001	Nil
30	0·01	Nil	Nil
		0·001	12·0
7	0·01	Nil	Nil
		0·01	8·1
	0·001	Nil	Nil
		0·01	6·1
	0·0001	Nil	Nil
		0·01	3·4
7	0·1	Nil	6·4
		1·0	8·6
		0·1	16·7
		0·01	13·3
		0·001	9·2

Note. In a series of 10 observations (inoculum 0·01 mg., citrate concentration 0·01 %), the mean growth in 7 days was 9·6 mg. (highest value 12·7, lowest 7·7).

(Carbon source, 1 % glucose; nitrogen source, $(NH_4)_2SO_4$; volume of medium, 20 ml.)

The optimal concentration for a short experiment was of the order 0·1–0·01 %; 1 % of citrate appears to inhibit the effect. It is noteworthy that an inoculum as small as 10^{-4} mg. can be stimulated to grow in 7 days by the presence of 0·01 % of citrate.

The experiments of Table 5 show that the citrate effect continues undiminished through serial subcultures. The organisms were inoculated as a washed suspension prepared in the usual manner. Three flasks were set up at each transfer: a control without citrate, and two containing 0·01 % of citrate. One of the resulting cultures was weighed and the other used for the next transfer.

Table 5. *Serial transfer of* Myco. phlei *in a synthetic medium*

	Growth (mg. dry wt.) with	
	Control (no citrate)	0·01 % citrate
Original culture	Nil	8·3
1st subculture	Nil	8·7
2nd ,,	Nil	8·7
3rd ,,	Nil	8·0
4th ,,	Nil	8·5
5th ,,	Nil	9·5, 9·5 (duplicates)

(Carbon source, 1 % glucose; nitrogen source, $(NH_4)_2SO_4$. Time of incubation, 7 days; inoculum, 0·01 mg. wet wt.)

The following substances related to or derived from citric acid were entirely devoid of stimulant action, when used in equivalent concentrations ($3{\cdot}9 \times 10^{-4} M$): Tricarballylic acid, aconitic acid (*trans*), trimethyl citrate, citramide, acetyl-citric acid, *dl*-citramalic acid, itaconic acid, citraconic acid, citrazinic acid, citrazinamide, and *iso*-nicotinic acid. The last three were tried because it was thought possible that ammonia might condense with citric acid to give a pyridine derivative as an intermediary metabolite.

The citric acid (initially 1·49 mg. in 20 ml.) remaining in the medium after 7 days was determined by the method of Pucher, Sherman & Vickery [1936]: about one-half of the citric acid had disappeared.

Table 6. *The stimulant effect of amino-acids on the growth of* Mycobacteria *in a synthetic medium*

Substance added (0·01 %)	Growth (mg. dry wt.) with *Myco. phlei*	*Myco. smegmatis*	*Myco. stercoris*	*Myco. Kedrowsky*	*Myco. Karlinski*	*Myco. ranae*
	(*a*) Source of carbon, 1 % glucose					
Syn. *dl*-Alanine	11·8	13·2	8·0	0·5	8·0	Trace
Syn. *dl*-Valine	10·4	9·1	7·7	—	6·4	Trace
Syn. *dl*-Leucine	13·6	6·6	5·4	0·7	5·6	Trace
Syn. *dl*-Norleucine	8·1	6·2	15·7	0·5	5·6	Trace
Syn. *dl*-Glutamic acid	10·6	31·6	13·9	0·8	7·3	Trace
l(−)-Tryptophan	11·0	8·5	1·6	0·2	10·3	Trace
l(−)-Proline	10·2	2·6	1·2	0·5	9·5	Trace
Syn. β-Alanine	Nil	Nil	Nil	Nil	Nil	Nil
Syn. *dl*-β-Amino-*n*-butyric acid	Nil	Nil	Nil	Nil	Nil	Nil
Syn. *dl*-δ-Amino-*n*-valeric acid	Nil	Nil	Nil	Nil	Nil	Nil
None	Nil	Nil	Nil	Nil	Nil	Nil
	(*b*) Source of carbon, 1 % glycerol					
Syn. *dl*-Alanine	0·5	17·2	—	—	—	—
Syn. *dl*-Glutamic acid	1·0	13·5	6·3	—	—	—
Syn. *dl*-Leucine	0·1	—	—	—	—	—
l(−)-Proline	—	15·4	—	—	—	—
None	Nil	Nil	Nil	—	—	—

Note. (1) Controls in which the source of carbon was omitted showed no growth. (2) The prefix 'syn.' means 'synthetic'. (Main nitrogen source, $(NH_4)_2SO_4$. Time of incubation, 7 days. Inoculum, 0·01 mg. to 20 ml. of medium in all cases except *Myco. ranae*, with which the inoculum was reduced to 0·00001 mg.)

Table 7. *The stimulant effect of amino-acids on the growth of* Myco. phlei

Addition to medium: Substance	Conc. (%)	Growth (mg. dry wt.) with source of carbon as 1 % of: Glucose	Succinate
None	—	Nil	Nil
Syn. *dl*-Alanine	0·02	11·8	1·7
l(+)-Alanine	0·01	11·5	—
Syn. β-Alanine	0·01	Nil	Nil
Syn. *dl*-Serine	0·01	11·2	—
l(−)-Cystine	0·01	2·8	—
Syn. *dl*-Phenylalanine	0·02	10·3	—
l(−)-Phenylalanine	0·01	9·2	—
d(+)-Phenylalanine	0·01	9·2	—
l(−)-Tyrosine	0·01	8·9	—
Syn. *dl*-α-Amino-*n*-butyric acid	0·01	7·4	—
Syn. *dl*-Threonine	0·01	Trace	—
Syn. *dl*-Methionine	0·01	9·1	—
Syn. *dl*-Leucine	0·01	13·6	Trace
Syn. *dl*-Isoleucine	0·01	7·0	—
Syn. *dl*-Lysine	0·01	12·0	—
Syn. *dl*-Aspartic acid	0·01	11·6	1·9
l(−)-Asparagine	0·01	11·7	—
Syn. *dl*-Glutamic acid	0·01	10·6	2·4
l(+)-Glutamine	0·01	7·1	—
Syn. *dl*-α-Amino-*n*-caprylic acid	0·01	10·5	Trace
l(+)-Arginine	0·01	7·3	—
l(+)-Ornithine	0·01	3·5	—
l(−)-Histidine	0·01	9·8	—
l(−)-Proline	0·01	10·2	Trace
l(−)-Hydroxy-proline	0·01	9·1	—
Glycine	0·01	9·7	—
Glycyl-glycine	0·01	9·2	—
Glycyl-*l*(−)-leucine	0·01	3·6	—
l(−)-Leucyl-glycine	0·01	15·0	—
Glutathione	0·01	8·6	—
Casein	0·01	7·5	—
Peptone (Witte)	0·01	10·3	—

Note. Controls in which the carbon source was omitted gave no growth.
(Main source of nitrogen, $(NH_4)_2SO_4$. Time of incubation, 7 days. Inoculum, 0·01 mg. to 20 ml. of medium.)

The effect of amino-acids, peptides and proteins. An effect similar to that of citrate is produced by small concentrations of amino-acids, dipeptides and proteins. The results are given in Tables 6 and 7.

Although amino-acids are not essential nutrients, growth is promoted by α-amino-acids, which are constituents of protein, and in which the α-amino group is free or bound in peptide linkage. The active agent appears to be either the whole amino-acid molecule, or the part of it represented by the grouping $=C(NH_2)—COOH$, since this is the only structure common to the 23 amino-acids which induced growth. There is no significant difference between the activity of those stereo-isomers that could be tested; the effect varies quantitatively, and is least with *dl*-threonine. Proline and hydroxyproline act like the α-amino-acids. Since these are protein constituents and probably convertible into α-amino-acids during metabolism, their stimulant action is not regarded as exceptional. No special importance is attached to the effect of crude proteins (peptone and casein); or to that of glutathione, since spontaneous decomposition into cysteinylglycine and pyrrolidone carboxylic acid may occur during 7 days' incubation at 38°.

When the free amino group is blocked by acetylation or benzoylation, conjugated with cholic acid, or otherwise substituted, the stimulant action is lost. β-Amino-acids, and others in which the amino group is displaced still farther from the carboxyl group, are also inactive.

Myco. phlei was tested more thoroughly than the other organisms. The following additional substances (concentration 0·01 %) failed to stimulate the growth of *Myco. phlei* subsisting on glucose: (1) sarcosine, 2:5-di-keto-piperazine, N-benzoyl-glycine, glycocholic acid, N-acetyl-alanine, N-benzoyl-alanine, N-phenyl-glycine, α-amino-phenyl-acetic acid, *dl*-ε-amino-caproic acid, *dl*-α-amino-*iso*-butyric acid, *dl*-α-amino-α-methyl-*n*-butyric acid; (2) urea, guanidine, creatine, taurine, glucosamine, and succinimide.

The effect of histamine. When a number of amines, amides and heterocyclic bases was tested, the only one that proved to be active was histamine. The magnitude of the effect is shown in Table 8.

The following were negative: (1) tyramine, tryptamine, putrescine, betaine, choline, ethanolamine, ethylamine, propylamine, *n*-butylamine, and *sec.*-octylamine; (2) acetamide, propionamide, *n*-butyramide, *n*-caproamide, and malonamide; (3) pyrrole, pyridine, pyrrolidine and piperidine. The concentration in each case was 0·01 %.

The effect of other organic substances. Many other organic substances were tested, but all were devoid of stimulant properties in so far as they produced no growth in 7 days. The organism used was *Myco. phlei*, and 0·01 mg. was seeded in 20 ml. of synthetic medium containing 1 % of glucose. Although it is unnecessary to list the whole series as negative, the fact that the following were ineffectual is recorded:

Table 8. *Stimulation of the growth of* Myco. phlei *by histamine*

Addition to medium: Substance	Conc. (%)	Bacterial growth (mg. dry wt.)
None	—	Nil
Histamine hydrochloride, synthetic (Eastman Kodak Co. Ltd.)	0·01	9·2
Histamine hydrochloride (British Drug Houses, Ltd.)	0·01	9·0
Histamine phosphate (British Drug Houses, Ltd.)	0·01	9·1
Sodium citrate	0·01	10·4

(Synthetic medium, volume 20 ml. Carbon source, 1 % glucose. Nitrogen source, $(NH_4)_2SO_4$. Inoculum, 0·01 mg. Incubation, 7 days.)

(1) Normal saturated fatty acids from C_1 to C_{18}, crotonic and oleic acids, β-hydroxy-*n*-butyric acid, *iso*-butyric and α-hydroxy-*iso*-butyric acids, normal chain dicarboxylic acids from C_2 to C_8, and hydroxy-malonic acid (concentrations, 0·01 %).

(2) Fructose monophosphate, arabinose, *d*-ribose, inositol, sorbitol, glycerol, ethylene glycol, ethanol, methanol, dihydroxy-acetone, gluconic, glyceric, lactic, pyruvic, *l*-malic, fumaric, *d*-tartaric and α-keto-glutaric acids (concentrations, 0·01 %).

(3) Uracil, adenine, adenosine, guanine, hypoxanthine, allantoin, alloxan, and hydantoin (concentrations, 0·01 %).

(4) Aneurin (100 μg.), riboflavin (50 μg.), nicotinic acid (100 μg.), phthiocol (20 μg.), pyridoxin (50 μg.), pantothenic acid (100 μg.), pyocyanine (500 μg.), cytochrome *c* (100 μg.), haemin (1 mg.), 2-methyl-1:4-naphthoquinone (saturated), ascorbic acid, indole acetic acid, β-indole propionic acid, α-naphthalene acetic acid, *p*-aminobenzoic acid and nicotinamide (all 2 mg.). The quantities in brackets are the amounts present in 20 ml. of medium.

The effect of calcium and iron. It has been shown that certain *Mycobacteria* will grow in a synthetic medium containing an oxidizable source of carbon if the seeding is sufficiently large and the incubation time sufficiently long; and that the lag period can be shortened, and growth enhanced, by addition of citrate, α-amino-acids and histamine, in low concentrations. The basis of the synthetic medium is a neutral solution containing NH_4^+, K^+, Na^+, Mg^{++}, $SO_4^=$, $PO_4^{\equiv}$, Cl^-, HCO_3^- and $CO_3^=$. Since traces of calcium and iron are present as impurities in the reagents, the question arose as to whether the amounts of these, and possibly of other elements, were optimal for growth.

Hay ash, rich in Ca^{++} and Fe^{+++}, was prepared and used according to Fildes & Richardson [1935], the result being a considerable stimulation of growth. The separate effects of Ca and Fe were

also investigated: the addition of either in low concentration was followed by growth, the character of which depended on the element. Results with *Myco. phlei* as test organism are given in Table 9. Ca was added as chloride, Fe as ferrous sulphate or ferric chloride, the solutions being added aseptically to the final medium. Ca was also added as $CaCO_3$, before autoclaving, in quantity just sufficient to saturate the basal salt mixture.

In the presence of Fe, initially Fe^{++} or Fe^{+++}, *Myco. phlei* formed a pigmented granular bottom growth, later extending to the surface as a thin yellow film; but in the presence of Ca the bottom growth developed as a slimy, whitish deposit and the surface pellicle was white. The effective concentrations are given in Table 9, which also permits a comparison with the effects of citrate and hay ash, and shows that when Mg^{++} is not added to the basal salt mixture, the stimulant effect of citrate can still be evoked. Increasing the concentration of Mg^{++} to the point of precipitating $MgNH_4PO_4$ does not stimulate growth.

Table 9. *Effect of inorganic substances on the growth of* Myco. phlei

Addition to medium		
Material	Amount added to 20 ml. of medium	Bacterial growth (mg. dry wt.)
None	—	Nil
Sodium citrate	2 mg.	12·6
Hay ash	Equiv. to 1 mg. ash	7·8
Fe^{+++}	75 μg.	7·5
	50 μg.	1·8
	20 μg.	0·1
	10 μg.	Nil
Fe^{++}	50 μg.	1·5
Ca^{++}	100 μg.	7·6
	50 μg.	3·0
	20 μg.	Nil
$CaCO_3$	Saturated	7·0
Mg^{++}	50 μg.	Nil
Sodium citrate (Mg^{++} not added to basal mixture)	2 mg.	9·7

(Carbon source, 1 % glucose. Nitrogen source, $(NH_4)_2SO_4$. Inoculum, 0·01 mg. Time of incubation, 7 days.)

A control test using the ash of analytical sodium citrate gave negative results. Other ions were tested with negative results: Ni^{++}, Co^{++}, Mn^{++}, Cu^{++}, Cd^{++}, Zn^{++}, Sn^{++}, Al^{+++}, Ba^{++}, La^{+++}, Ce^{++++}, Th^{++++}, Tl^{+}, Br^{-}, I^{-}, all 50 μg./flask. Sodium silicate (2 mg.) also gave negative results.

Earlier, anomalous results were encountered in testing some possible growth stimulants, which were dissolved in the basal salt mixture and filtered through a Seitz pad. Growth occurred, although it was unexpected in view of other general findings. When the experiments were repeated by adding the unsterilized materials to autoclaved salt mixture, there was no growth. Attention was thus directed to the Seitz filtrate, which was slightly turbid and gave a test with ammonium oxalate indicating the presence of about 100 μg. of Ca^{++}/20 ml.; the Prussian blue test for Fe^{+++} [Feigl, 1937] revealed about 1–10 μg. of Fe^{+++}/20 ml., but the α-α'-dipyridyl test for Fe^{++} [Feigl, 1937] was negative. Subsequently it was discovered that both water and saline could extract detectable quantities of Ca^{++} and Fe^{+++} from filter pads in a single filtration. These findings are described elsewhere [Hunter, 1943], and it will suffice to state that they do not invalidate any observations recorded in this communication. The aseptic addition of a small volume of filtered solution (up to at least 2 ml.) to a synthetic medium, the final volume of which is 20 ml., does not convey sufficient Ca or Fe to affect the growth of *Mycobacteria* under the conditions we have employed.

Preliminary observations on the growth of tubercle bacilli and Myco. butyricum, *no.* 337

In studying the growth of tubercle bacilli (strains T_1 and H 37) in synthetic media we have found that small inocula do not propagate during an incubation period of 6 weeks. It was necessary to use either 5 mg. of washed suspension or a washed pellicle large enough to cover a 2 mm. loop. Mother cultures were 3 weeks old. Growth was examined in media containing 1 % of one of glycerol, glucose or *dl*-lactate, with and without addition of hay ash, citrate and amino-acids, separately and together. The surface of glycerol-broth medium in the control tubes was covered within 3 weeks, but growth was poor in the synthetic media, amounting to no more than a thickening of the inoculum or a slight patchy extension. Growth could be stimulated to normal dimensions by addition of small quantities of Marmite or muscle *Kochsaft*.

It has been seen that a small inoculum of *Myco. butyricum* grows poorly in the synthetic medium. Larger inocula in the form of washed surface pellicles also failed to exhibit extension in a glycerol medium to which hay ash, citrate, and amino-acid mixtures had been added. Again muscle *Kochsaft* and Marmite stimulated rapid growth approaching that of broth controls.

These observations suggest that the tubercle bacillus and *Myco. butyricum* (no. 337) have a more exacting nutrition than the other organisms studied.

DISCUSSION

When certain species of *Mycobacteria* are lightly seeded in a synthetic medium under the conditions described in this paper, it is possible to demonstrate

the effectiveness of growth stimulants which operate in concentrations of the order $1 \times 10^{-3} M$ or lower. The active substances belong to four apparently independent classes: (1) citric acid, (2) α-amino-acids, (3) histamine, (4) ions of calcium and iron. They are termed 'growth stimulants' because they do not conform to the definition of essential growth factors [Fildes & Richardson, 1935].

Many investigators have found that citric acid is an advantageous constituent of media specially designed for cultivation of tubercle bacilli. Under the conditions of our experiments low concentrations of citric acid do not stimulate the growth of tubercle bacilli, but this substance effectively reduces the lag period and promotes the growth of acid-fast saprophytes. Experiments with *Myco. phlei* show that citric acid is not oxidized as a source of energy; nor does it catalyze the oxidation of carbohydrate after the manner of the citric acid cycle [Krebs & Johnson, 1937], since intermediates of the cycle—such as α-keto-glutaric, succinic, fumaric and *l*-malic acids—do not stimulate growth.

Although citric acid is not oxidized, it disappears from the medium. It is possible that the organisms normally synthesize small amounts of citric acid from a variety of sources of carbon during the lag period. If this were so, the stimulant effect of citric acid would be explained.

The stimulant properties of amino-acids have been observed with other bacterial families, e.g. *Esch. coli* [Sahyun, Beard, Schultz, Snow & Cross, 1936] and propionic acid bacteria [Wood, Anderson & Werkman, 1938]. The possibility that citric acid is concerned in the synthesis of amino-acids was considered, but no evidence to support the suggestion was obtained.

Sauton [1912] and Long & Seibert [1926] found that Fe enhances the growth of tubercle bacilli. Our experiments show that the growth of acid-fast saprophytes can be stimulated by very low concentrations of Ca and Fe; but in the absence of added Ca^{++}, Mg^{++} or Fe^{+++} growth still occurs if citrate be present, showing that if any, or all, of these ions be essential, sufficient is provided by the impurities of analytical reagents.

It must be emphasized that the *Mycobacteria* used in this work have been cultivated on laboratory media for years and have had ample opportunity to adapt themselves to artificial conditions. Although the stock cultures have been kept on a rich medium, it is possible that freshly isolated organisms would have more exacting nutritional demands.

SUMMARY

1. The respiration of certain species of *Mycobacteria* has been determined in presence and absence of substrates. Species vary widely with respect to the respiration of 'resting' cells and to oxygen consumption in the presence of different substrates.

2. Growth of small inocula in a synthetic medium has been studied. It was found that under defined conditions the growth of some species is stimulated by citric acid, α-amino-acids, histamine, calcium and iron.

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The Antibacterial Action of some Stilbene Derivatives

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Early types of antibacterial substances were not specifically antibacterial, but were usually toxic to all living cells, and were of value only in so far as they could be employed without serious damage to the host. The search for more suitable antibacterial agents resulted in the preparation and testing of many substances of widely different constitution, and it was from the empirical yet systematic investigations of one such class of chrysoidine dyestuff that knowledge of the activity of sulphanilamide arose. The growing evidence that the antibacterial action of sulphanilyl drugs was due to competition with an enzymic metabolic process suggested to Fildes [1940] a new approach to the problem. This was the design of antibacterial agents by the synthesis of compounds with a close spatial resemblance to substances vital to, or closely associated with, bacterial growth and reproduction. These growth substances include the water-soluble vitamins of the animal world, which now appear to be essential to the life of all growing cells. The method has been recently used in a sufficiently diverse series of independent investigations to confirm the principle [McIlwain, 1943].

Other compounds which exert a pronounced physiological activity in minute concentration are the animal hormones related to cyclopentanophenanthrene; for example, oestradiol, when injected into an ovariectomized rat of 50 g. body weight in a dose of 0·2 μg., is capable of doubling the weight of the uterus; with a blood volume of 2 ml., this represents an activity at a concentration of 1 in 10^7. These compounds are not known to be hormones for the vegetable kingdom, but they are widely distributed in nature and are probably constituents of all growing cells, being found in all plants and bacteria [Aschheim & Hohlweg, 1933], as well as in all animals. It seems to us that these compounds can readily be incorporated into the living cell, and that such molecules constitute suitable material which, while not necessarily competitive with known essential systems, is capable of modification to contain known antibacterial groupings. Synthetic work in the cyclopentanophenanthrene series is difficult, and it is fortunate that the work of Dodds, Golberg, Lawson & Robinson [1939] provided, in the diethylstilboestrols, simple substances with space configurations similar to those of the naturally occurring compounds, and with their intense physiological activity.

We have prepared and tested a number of compounds in this series for antibacterial properties and have found some of them very powerfully bactericidal. As with the oestrogenic properties of compounds in this series, the antibacterial activities vary considerably with the structure of the molecule, though these variations are not altogether analogous. The compounds tested are set out in Table 1 and fall into seven main classes:

I. Deoxystilboestrol and its dihydro-derivative (1 and 2).

II. α:α′-Diethylstilbene, intermediates in its preparation and some derivatives (3–7).

III. Deoxydiethylstilboestrol, intermediates and derivatives (8–14).

IV. Diethylstilboestrol and derivatives (15–20).

V. Amino-, cyano-, amidino- and nitro-derivatives (21–29).

VI. Naturally occurring steroids (31 and 32).

VII. Penicillic acid and citrinin (33 and 34).

I–VI are poorly soluble in water and in broth; therefore saturated solutions in broth were first prepared by making use of the solubility of the compounds in 90 % (v/v) ethanol, in which a 10 % solution can be prepared. Small volumes of this solution, containing a known amount of drug, were added to measured volumes of nutrient broth and the mixture autoclaved. When a volume of 0·1 ml. was added to 100 ml. of broth, the material remained in solution, or in a very finely divided suspension, at 37°, and from the starting solution or suspension a series of concentrations of drug was made by pipetting 5 ml. into a suitable quantity of sterile broth. This series of drug broths was then inoculated with a suspension of the strains of *Staphylococcus* or *Streptococcus* used in these experiments. The inocula were adjusted to give a final count of 1000–2000 organisms/ml. of drug broth and the actual numbers present were determined by a plate count in each case.

Figures are given in Table 1 for the minimal concentration of drug which just prevents visible growth of the organism after 48 hr. incubation at 37°. Subcultures from tubes containing the minimal inhibitory concentration showed that the action of

Table 1. *Bactericidal activity of the compounds tested against strains of* Staphylococcus *and* Streptococcus

			Staphylococcus	*Streptococcus*
1.	4-OH.C_6H_4.CH=CH.C_6H_5	Hewitt *et al.* [1912]	1: 50,000	1: 50,000
2.	4-OH.C_6H_4.CH_2.CH_2.C_6H_5	Stoermer & Kippe [1903]*	1: 40,000	1: 20,000
3.	C_6H_5.CO.CHEt.C_6H_5	Janssen [1889]	1: 200,000	1: 200,000
4.	C_6H_5.C(OH)Et.CHEt.C_6H_5		1: 30,000	1: 30,000
5.	C_6H_5.CEt=CEt.C_6H_5	Kharasch & Kleiman [1943]*	1: 10,000	1: 60,000
6.	C_6H_5.CHEt.CHEt.C_6H_5	Kharasch & Kleiman [1943]*	Inactive	Inactive
7.	C_6H_5.C(OH)Et.C(OH)Et.C_6H_5	Ciamician & Silber [1914]	Slight bacteriostatic activity	Inactive
8.	4-MeO.C_6H_4.CO.CHEt.C_6H_5	Dodds *et al.* [1939]	Inactive	Inactive
9.	4-MeO.C_6H_4.C(OH)Et.CHEt.C_6H_5	Dodds *et al.* [1939]	Inactive	Inactive
10.	4-MeO.C_6H_4.CHEt.CHEt.C_6H_5		Inactive	Inactive
11.	4-OH.C_6H_4.CEt=CEt.C_6H_5	Dodds *et al.* [1939]*	1:640,000	1:2,000,000
12.	4-OH.C_6H_4.CHEt.CHEt.C_6H_5		1:200,000	1: 800,000
13.	4-BzO.C_6H_4.CHEt.CHEt.C_6H_5		Inactive	Inactive
14.	4-HSO_3O.C_6H_4.CHEt.CHEt.C_6H_5		1: 3,000	1: 640,000
15.	4-OH.C_6H_4.CEt=CEt.C_6H_4.OMe-4′		1: 10,000	1: 20,000
16.	4-OH.C_6H_4.CEt=CEt.C_6H_4.OH-4′ (h.m.†)	Dodds *et al.* [1939]	1:100,000	1: 400,000
17.	4-OH.C_6H_4.CEt=CEt.C_6H_4.OH-4′ (l.m.‡)	Dodds *et al.* [1939]	1:100,000	1: 400,000
18.	4-OH.C_6H_4.CHEt.CHEt.C_6H_4.OH-4′ (h.m.)	Dodds *et al.* [1939]	1:100,000	1: 300,000
19.	4-OH.C_6H_4.CHEt.CHEt.C_6H_4.OH-4′ (l.m.)	Dodds *et al.* [1939]	1: 80,000	1: 400,000
20.	4-AcO.C_6H_4.CHEt.CHEt.C_6H_4.OAc-4′ (l.m.)	Dodds *et al.* [1939]	1: 50,000	1: 100,000
21.	C_6H_5.CO.CHEt.C_6H_4.NH_2-4		1: 8,000	1: 8,000
22.	C_6H_5.C(OH)Et.CHEt.C_6H_4.NH_2-4		1: 5,000	1: 10,000
23.	C_6H_5.CEt=CEt.C_6H_4.NH_2-4		1: 32,000	1: 128,000
24.	C_6H_5.CEt=CEt.C_6H_4.$NHSO_2$.C_6H_4.NH_2-4		1: 40,000	1: 40,000
25.	4-MeO.C_6H_4.CH=CH.C_6H_4.NO_2-4′	Hewitt *et al.* [1912]	1: 10,000	1: 10,000
26.	4-OH.C_6H_4.CH=CH.C_6H_4.NO_2-4′	Cullinane [1923]	1: 20,000	1: 20,000
27.	4-OH.C_6H_4.CH=CH.C_6H_4.NH_2-4′		Inactive	Slight activity
28.	4-OH.C_6H_4.CH=CH.C_6H_4.CN-4′		Inactive	Slight activity
29.	4-OH.C_6H_4.CH=CH.C_6H_4.C(=NH)(NH_2)-4′		1: 30,000	1: 30,000
30.	4-OH.C_6H_4.CO.CHOH.CH_3	Auwers & Noll [1938]	1: 600	1: 600
31.	Oestrone		1: 33,000	1: 33,000
32.	Oestradiol		1: 15,000	1: 40,000
33.	Penicillic acid	Oxford [1942]	1: 1,500	—
34.	Citrinin	Oxford [1942]	1: 15,000	—

* Revised method of preparation given. † h.m. = high-melting. ‡ l.m. = low-melting.

the drugs was usually bactericidal, but bacteriostasis was also shown in some cases at lower concentrations by the appearance of delayed growth 24–48 hr. after inoculation.

When a compound is stated in Table 1 to be inactive, it indicates that a saturated solution obtained by filtering broth suspension containing the drug in a concentration of 1/100,000 failed to prevent the growth of a small number of organisms.

The antibacterial activities of deoxydiethylstilboestrol and its dihydro-derivative were tested against a number of gram-negative organisms, but both compounds were generally inactive in the highest concentrations obtainable.

So that our results may be comparative we have included in the series estimates of penicillic acid and citrinin; with our strain of *Staphylococcus* penicillic acid is bactericidal at a concentration of 1:1500 and citrinin at 1:15,000, so that our most active specimen, 4-OH.C_6H_4.CEt=CEt.C_6H_5 (11), is some 40 times as active as the latter.

Deoxydiethylstilboestrol is soluble in soap solutions and its germicidal action may be compared with soap solutions of the chlorinated phenols. The results obtained in a Rideal-Walker type test, where in addition to *Eberthella typhosa*, strains of *Streptococcus*, *Staphylococcus*, and *Pseudomonas aeruginosa* (*Ps. pyocyanea*) were examined, are given in Table 2. The minimal concentration required to kill gives the concentration of disinfectant in which life

Table 2. *Comparative bactericidal properties of phenol, chlorinated phenols and deoxydiethylstilboestrol*

	Minimal concentration required to kill			
Material*	*Streptococcus pyogenes* (P 90)	*Staphylococcus aureus* (133)	*Ps. aeruginosa* (1)	*Eberthella typhosa* (Rawlings)
1% deoxydiethylstilboestrol	1:1600	1: 5	1:10	1: 2·2
1% *p*-chloro-*m*-xylenol	1:1600	1: 5	1:10	1:27
1% *p*-chloro-*m*-cresol	1: 800	1: 2·5	1:10	1:20
5% phenol	1: 120	1:80	1:70	1:90

* The compounds were dissolved in 5% sodium oleate solution.

survives for 2½ and 5 min. but not thereafter. Deoxydiethylstilboestrol is as effective a disinfectant as chloroxylenol against gram-positive organisms but is less effective against gram-negative types.

Some of the compounds were tested in mice infected with virulent strains of staphylococci and streptococci, the drugs being administered by mouth in three doses, immediately, 5 hr., and 24 hr. after infection. The dose was 1–10 mg./g. of mouse, and no protection was afforded by any of the compounds tried, with the possible exception of

4-HSO_3O.C_6H_4.CHEt.CHEt.C_6H_5 (14)

which showed very slight activity in this respect.

DISCUSSION

The comparison of the bactericidal activities presented in the table shows that the two most active compounds are deoxydiethylstilboestrol (11) and its dihydro-derivative (12). The factors which appear to effect the activity of the compounds tested are: (*a*) the carbon skeleton; (*b*) the substituent groups; (*c*) the degree of unsaturation of the central bridge.

The preferred spatial configuration of the diethylstilbene type as compared with the unalkylated stilbene type is shown by a comparison of

4-OH.C_6H_4.CH=CH.C_6H_5 (1)—1:50,000,

and

4-OH.C_6H_4.CEt=CEt.C_6H_5 (11)—1:640,000;
4-OH.C_6H_4.CH_2.CH_2.C_6H_5 (2)—1:40,000,

and

4-OH.C_6H_4.CHEt.CHEt.C_6H_5 (12)—1:200,000.

(The ratios here and in subsequent comparisons refer to the minimal bactericidal dilutions (Table 1) of these compounds against *Staphylococcus*.)

The effect of the hydroxy group in the 4-position is shown by the pairs:

C_6H_5.CEt=CEt.C_6H_5 (5)—1:10,000,

and

4-OH.C_6H_4.CEt=CEt.C_6H_5 (11)—1:640,000;
C_6H_5.CHEt.CHEt.C_6H_5 (6)—inactive,

and

4-OH.C_6H_4.CHEt.CHEt.C_6H_5 (12)—1:200,000.

This evidence is of interest in that bactericidal activity and oestrogenic activity both require the presence of the 4-hydroxy group.

However, analogy with oestrogenic activity ends here, since the reduction of the centre double-bond diminishes, not enhances, the antibacterial activity, e.g.

4-OH.C_6H_4.CH=CH.C_6H_5 (1)—1:50,000,

and

4-OH.C_6H_4.CH_2.CH_2.C_6H_5 (2)—1:40,000;
C_6H_5.CEt=CEt.C_6H_5 (5)—1:10,000,

and

C_6H_5.CHEt.CHEt.C_6H_5 (6)—inactive;
4-OH.C_6H_4.CEt=CEt.C_6H_5 (11)—1:640,000,

and

4-OH.C_6H_4.CHEt.CHEt.C_6H_5 (12)—1:200,000;

and the introduction of a hydroxyl into the second ring diminishes the activity:

4-OH.C_6H_4.CEt=CEt.C_6H_5 (11)—1:640,000,

and

4-OH.C_6H_4.CEt=CEt.C_6H_4.OH-4′ (16 and 17)—1:100,000;
4-OH.C_6H_4.CHEt.CHEt.C_6H_5 (12)—1:200,000,

and

4-OH.C_6H_4.CHEt.CHEt.C_6H_4.OH-4′ (18 and 19)—1:100,000 and 1:80,000.

The two forms of diethylstilboestrol (16 and 17) have similar antibacterial activities, whereas the normal form is about 14 times as potent an oestrogen as ψ-stilboestrol. Thus while both molecules may be readily accepted by cells, both may prove bactericidal while only one is capable of fulfilling the oestrogenic function to a marked degree. (This is interesting if indirect evidence that the lack of oestrogenic activity of ψ-stilboestrol is not due to any 'hindrance' effect of the cell membrane.) Furthermore, high-melting hexoestrol (18) is very much more active oestrogenically than its lower melting stereoisomer (19), but their antibacterial activities are very close (1:100,000 and 1:80,000 respectively).

Conversion of the 4-hydroxyl to methoxyl destroys the activity completely, as shown by the inactivity of 4-MeO.C_6H_4.CHEt.CHEt.C_6H_5 (10); if there are two substituent hydroxyls, conversion of one of them to methoxyl greatly reduces the activity:

4-OH.C_6H_4.CEt=CEt.C_6H_4.OH-4′ (16)—1:100,000,

and

4-OH.C_6H_4.CEt=CEt.C_6H_4.OMe-4′ (15)—1:10,000;

cf. also

4-OH.C_6H_4.CH=CH.C_6H_4.NO_2-4′ (26)—1:20,000,

and

4-MeO.C_6H_4.CH=CH.C_6H_4.NO_2-4′ (25)—1:10,000.

The influence of hydroxy groups in the centre of the molecule is interesting.

$C_6H_5.CO.CHEt.C_6H_5$ (3)

which, in its enolic form, is

$C_6H_5.C(OH)=CEt.C_6H_5$,

is bactericidal at 1:200,000;

$C_6H_5.C(OH)Et.CHEt.C_6H_5$ (4),

which contains a hydroxyl but no double bond, at 1:30,000; the analogous hydrocarbons

$C_6H_5.CEt=CEt.C_6H_5$ (5)

and $C_6H_5.CHEt.CHEt.C_6H_5$ (6)

are bactericidal at 1:10,000, and inactive, respectively. The provision of a second hydroxyl in the centre, cf.

$C_6H_5.C(OH)Et.C(OH)Et.C_6H_5$ (7),

lowers the activity.

In the 4-amino series the effect is not quite the same. Here compounds containing the hydroxyl in the centre are less active than the parent substance, though the activity is still greater in presence of a double bond. Thus

$C_6H_5.CO.CHEt.C_6H_4.NH_2$-4 (21)

is active at 1:8000 and

$C_6H_5.C(OH)Et.CHEt.C_6H_4.NH_2$-4 (22)

at 1:5000. However, the compound

$C_6H_5.CEt=CEt.C_6H_4.NH_2$-4 (23)

is active at 1:32,000, an increase possibly to be ascribed to the amino group here freed from any interference by the hydroxyl.

Since in the diethylstilboestrol series the maximal oestrogenic activity appears to be associated with compounds containing two hydroxyls in the 4:4′-positions, it seemed possible that some group present in the 4′-position might enhance the activity of 4-hydroxystilbene. Accordingly, a series of 4-hydroxystilbenes were prepared with the groups

—CN, $—NH_2$, $—C\begin{matrix}=NH\\ \diagdown NH_2\end{matrix}$ and $—NO_2$

(27, 28, 29 and 26 respectively) in the 4′-position. However, the last two depressed the activity slightly, whilst the first two practically destroyed it.

CHEMICAL METHODS

4-*Hydroxydibenzyl* (2). This compound was prepared by a more convenient method than that given in the literature [Stoermer & Kippe, 1903; Freund & Remse, 1890; Späth, 1913]. 4-Methoxydeoxybenzoin (7 g.) was refluxed with amalgamated zinc (30 g.), conc. HCl (100 ml.) and water (100 ml.) for 5 hr., fresh HCl being added each hour. The hot liquid was decanted from the zinc, cooled, the solid product collected, washed with water and crystallized from ethanol. 4-Methoxydibenzyl was obtained in colourless plates, m.p. 61–62°, as in the literature. For hydrolysis this ether (6·3 g.) was dissolved in an ethereal solution of methyl magnesium iodide (prepared from 0·8 g. of magnesium, and 4·0 ml. of methyl iodide in 10 ml. of ether). The mixture was evaporated to dryness and heated in an oil-bath until frothing started (*c.* 180°); as this decreased the temperature was gradually raised to 200°. After 3 hr. the mixture was decomposed with ice and HCl and the colourless insoluble product filtered, washed and crystallized (as the sodium salt) from hot aqueous 2*N* NaOH. The regenerated 4-hydroxydibenzyl crystallized from carbon tetrachloride in colourless plates (4 g.), m.p. 100–101° as in the literature.

α:β-Diethyl-α-hydroxydibenzyl (4). α-Ethyldeoxybenzoin (14·0 g.) was dissolved in ether (50 ml.) and gradually treated with a solution of ethyl magnesium bromide (from 2 g. of magnesium and 7 ml. of ethyl bromide in 30 ml. ether). The addition completed, the mixture was heated on a water-bath for 10 min., cooled, and the product decomposed with ice and HCl. The ethereal layer was separated, dried, the solvent removed and the residue distilled *in vacuo*, b.p. 185–195° at 18 mm. (13 g.). On redistillation, α:β-diethyl-α-hydroxydibenzyl was obtained as a colourless viscous oil, b.p. 182–186° at 14 mm. (Found: C, 85·0; H, 8·6%. $C_{18}H_{22}O$ requires C, 85·0; H, 8·7%.)

α:β-Diethylstilbene (5). α:β-Diethyl-α-hydroxydibenzyl was dehydrated with phosphorus trichloride [Dodds *et al.* 1939] giving α:β-diethylstilbene as a colourless mobile oil, b.p. 170° at 15 mm. (Found: C, 91·2; H, 8·4%. Calc. for $C_{18}H_{20}$, C, 91·5; H, 8·5%.) This is in agreement with Ramart-Lucas & Anagostopoulos [1928] and also with Kharasch & Kleiman [1943], but not with Rising & Zee [1928] or Carlisle & Crowfoot [1941], who describe it as a crystalline solid.

α:β-Diethyldibenzyl (6). Diethylstilbene (5·6 g.) was shaken in an atmosphere of hydrogen with platinum oxide (0·5 g.) and acetone (50 ml.). The solution was filtered, evaporated, and the residue (4·5 g.; m.p. 74–76°) recrystallized from methanol. α:β-Diethyldibenzyl was obtained in long needles, m.p. 83–84°. (Found: C, 90·7; H, 9·1%. Calc. for $C_{18}H_{22}$, C, 90·7; H, 9·3%.) This melting-point is much lower than values recorded in the literature. Moritz & Wolfenstein [1899] give 88°; Späth [1913] gives 92–93°.

γ:δ-Dihydroxy-γ:δ-diphenylhexane (7). Propiophenone (6 g.) in wet ether (100 ml.) was treated with aluminium amalgam (prepared from 6 g. aluminium powder and 9 g. mercuric chloride in 300 ml. ethanol) and, when the reaction had subsided, the mixture was refluxed on a water-bath for 45 min. The ether was evaporated and the residue crystallized repeatedly from light petroleum (b.p. 40–60°) yielding a white solid, m.p. 135–136° (Ciamician & Silber [1914] give 138–139°).

4-*Methoxy-α:β-diethyldibenzyl* (10). 4-Methoxy-α:β-diethylstilbene (20 g.) was shaken in acetone (100 ml.) with palladium charcoal catalyst (prepared by reducing 0·2 g. palladium chloride in presence of 3·8 g. charcoal) in hydrogen at 1 atm. pressure. After filtration the solution was evaporated and the product crystallized from ethanol; it had m.p. 89–90° (19 g.). (Found: C, 84·6; H, 8·8%. $C_{19}H_{24}O$ requires C, 85·0; H, 9·0%.)

4-*Methoxy-α:β-diethylstilbene.* δ-Phenyl-γ-anisylhexan-γ-ol, without purification, distilled at ordinary pressure with elimination of water, at 325–330°. The product, crystallized from ethanol, had m.p. 79–80°. (Found: C, 85·5; H, 8·2%. $C_{19}H_{22}O$ requires C, 85·7; H, 8·3%); the yield was quantitative.

4-*Hydroxy-α:β-diethylstilbene* (11). To a solution of methylmagnesium iodide (prepared from 5 g. of magnesium and

12 ml. of methyl iodide in 200 ml. of dry ether) was added 4-methoxydiethylstilbene (20 g.), and after evaporation of the ether the mixture was heated in an oil-bath, first at 170° until frothing subsided, and then at 200° for 30 min. After decomposition with water and HCl, the product was extracted with carbon tetrachloride and the solution concentrated to crystallize; m.p. 125–127°. (Found: C, 84·9; H, 8·0%. Calc. for $C_{18}H_{20}O$, C, 85·7; H, 8·0%.)

4-Hydroxy-α:β-diethyldibenzyl (12). To a solution of methylmagnesium iodide (prepared from 7·5 g. of magnesium and 18 ml. of methyl iodide in 300 ml. of ether) was added 4-methoxydiethyldibenzyl (30 g.) and, after evaporation of the ether, the mixture was heated in an oil-bath, first to 160° and then, when the reaction had subsided, to 195° for 30 min. After decomposition with water and acidification with HCl, the precipitate was crystallized from carbon tetrachloride; m.p. 139–140° (15 g.). (Found: C, 85·1; H, 8·7%. $C_{18}H_{22}O$ requires C, 85·0; H, 8·7%.) The *benzoate* (13), prepared by the Schotten-Baumann method or by the action of benzoyl chloride on a pyridine solution of the phenol, crystallized from methanol in long prisms, m.p. 110°. (Found: C, 83·8; H, 7·1%. $C_{25}H_{26}O$ requires C, 83·8; H, 7·3%.)

The *sulphonate* (14) was obtained as its pyridine salt by the action of chlorosulphonic acid (1·5 ml.) on a pyridine solution of the parent phenol (1·0 g.). As it was somewhat unstable to heat, it was purified by dissolution in cold chloroform and precipitation with ligroin. Colourless plates, m.p. 195–196°. (Found: C, 66·3; H, 6·4; S, 7·8; N, 3·5%. $C_{23}H_{27}O_4NS$ requires C, 66·8; H, 6·5; S, 7·7; N, 3·4%.)

Diethylstilboestrol monomethyl ether (15) was obtained as a by-product in the production of diethylstilboestrol by hydrolysis of the dimethyl ether. It crystallized from ethanol; m.p. 101–102°. (Found: C, 80·4; H, 7·7%. $C_{19}H_{22}O_2$ requires C, 80·8; H, 7·8%.)

4-Nitro-α-ethyldeoxybenzoin. 4-Nitrodeoxybenzoin [Petrenko-Kritschenko, 1892; Womack, Campbell & Dodds, 1938] (35 g.), was added to a hot solution of sodium (3·9 g.) in absolute ethanol (800 ml.), ethyl iodide (39 ml.) was added as quickly as possible to the resulting solution, and the whole refluxed until the solution was neutral (2–3 hr.). During this time the colour changed from deep purple to yellow. After pouring into water the solution was extracted with benzene, the benzene layer washed twice with water, dried over calcium chloride, and concentrated to *c.* 100 ml. This solution was treated with an equal volume of light petroleum (b.p. 60–80°), when a crystalline solid separated (31 g., m.p. 75–78°). Recrystallized from ethanol (charcoal), *4-nitro-α-ethyldeoxybenzoin* formed almost colourless prisms, m.p. 78–80°. (Found: C, 71·2; H, 5·9; N, 5·4%. $C_{16}H_{15}O_3N$ requires C, 71·4; H, 5·6; N, 5·2%.)

4-Amino-α-ethyldeoxybenzoin (21). 4-Nitro-α-ethyldeoxybenzoin (8 g.), iron filings (24 g.) and water (8 ml.) were ground into a paste, transferred to a flask together with xylene (8 ml.) and 1% $FeCl_3$ solution (12 ml.) and heated on a vigorously boiling water-bath for 3 hr., with continuous stirring. After 1½ hr., more xylene (4 ml.) was added. The mixture was allowed to cool somewhat, treated with benzene (100 ml.) and the iron oxide filtered off and washed thoroughly with a further quantity of warm benzene (100 ml.). The combined benzene layers were separated from the aqueous portion and exhaustively extracted with *N* HCl. By this means coloured resinous impurities remained in the benzene solution whilst, on making alkaline, the HCl deposited an oil which rapidly set to an almost colourless solid. This was collected, dried (6·6 g.) and crystallized (charcoal) from cyclohexane, from which *4-amino-α-ethyldeoxybenzoin* separated in sprays of needles, m.p. 128–129°. (Found: C, 80·5; H, 7·2; N, 6·0%. $C_{16}H_{17}ON$ requires C, 80·3; H, 7·1; N, 5·9%.)

4-Amino-α:β-diethyl-β-hydroxydibenzyl (22). A solution of 4-amino-α-ethyldeoxybenzoin (5·0 g.) in benzene (80 ml.) was gradually run into an ice-cooled solution of ethylmagnesium bromide (from 2 g. magnesium, 30 ml. ether and 7 ml. ethyl bromide, the ether being subsequently removed) in benzene (30 ml.). A thick yellow precipitate formed immediately. After standing overnight at room temperature the mixture was refluxed on a water-bath for 5 min., cooled and the magnesium complex decomposed with ice and ammonium chloride. A small amount of insoluble material was filtered off and the benzene layer separated, washed once with water and then extracted with HCl. The acid solution was made alkaline with ammonia, the resulting oil taken up in ether and the ethereal solution dried and evaporated. The crude residue crystallized on cooling and was purified by repeated recrystallization from a mixture of carbon tetrachloride and cyclohexane. Finally *4-amino-α:β-diethyl-β-hydroxydibenzyl* was obtained in colourless needles, m.p. 91–92°. (Found: C, 80·1; H, 8·3%. $C_{18}H_{23}ON$ requires C, 80·2; H, 8·6%.)

4-Amino-α:β-diethylstilbene (23). A solution of 4-amino-α:β-diethyl-β-hydroxydibenzyl (10 g.) in glacial acetic acid (30 ml.) was saturated with hydrogen chloride at room temperature. After 72 hr. it was diluted with conc. HCl (30 ml.) and after a further 72 hr. the thick, rather discoloured precipitate (8·7 g., m.p. 220–230°) was recrystallized (charcoal) from ethanol diluted with 4 vol. of 0·5*N* HCl. *4-Amino-α:β-diethylstilbene hydrochloride* was obtained in colourless needles, m.p. 254–255°. (Found: C, 75·2; H, 7·5%. $C_{18}H_{22}NCl$ requires C, 75·1; H, 7·7%.) The parent *base* regenerated in the usual fashion, crystallized from ligroin (b.p. 40–60°) in sprays of needles, m.p. 96–97°. (Found: C, 86·3; H, 8·4. $C_{18}H_{21}N$ requires C, 86·1; H, 8·4%.)

4-Sulphanilamido-α:β-diethylstilbene (24). Treatment of 4-amino-α:β-diethylstilbene in pyridine solution with 4-acetamidobenzenesulphonyl chloride gave *4-[-4″-acetamidobenzenesulphonamido]-α:β-diethylstilbene*. This crystallized readily from methanol in colourless needles, m.p. 207–208°. (Found: C, 69·5; H, 6·6. $C_{26}H_{28}O_3N_2S$ requires C, 69·6; H, 6·3%.)

On hydrolysis with HCl in ethanol, *4-sulphanilamido-α:β-diethylstilbene* was readily obtained. It crystallized from methanol or 95% ethanol in colourless needles, m.p. 180–182°. (Found: C, 70·9; H, 6·4; N, 7·15%. $C_{24}H_{26}O_2N_2S$ requires C, 70·9; H, 6·4; N, 6·9%.)

4-Hydroxy-4′-aminostilbene (27). A solution of 4-hydroxy-4′-nitrostilbene (1·0 g.) in ethanol (15 ml.) and concentrated ammonia (d. 0·880; 16 ml.) was gradually added to a boiling solution of $FeSO_4$ (11·0 g.) in water (75 ml.) and ethanol (150 ml.). The addition completed (1 hr.), the mixture was boiled for 1 hr. After cooling, the precipitated hydroxide was dissolved in dilute HCl, the yellow insoluble portion filtered off and crystallized from hot aqueous 2*N* NaOH. The crystalline sodium salt was collected and the parent phenol regenerated by treatment with dilute acetic acid. The crude *4-hydroxy-4′-aminostilbene* (0·75 g.) was a buff-coloured powder; it was sparingly soluble in most common organic solvents and could not be obtained quite pure. It crystallized from benzyl cyanide in bundles of yellowish plates, m.p. 270–271° (decomp.). (Found: C, 80·1; H, 6·2; N, 7·0%. $C_{14}H_{13}ON$ requires C, 79·6; H, 6·1; N, 6·6%.)

4-Hydroxy-4′-cyanostilbene (28). 4-Cyanophenylacetic acid [Jaeger & Robinson, 1941] (2·0 g.), 4-hydroxybenzaldehyde (1·0 g.) and piperidine (1 ml.) were heated at 140° for 1½ hr. The semi-liquid product was mixed with 50% ethanol, when a yellowish solid was rapidly deposited. This was collected, washed with fresh 50% ethanol, crystallized twice from glacial acetic acid and finally from 70% ethanol: *4-hydroxy-4′-cyanostilbene* was obtained in faintly coloured prisms, m.p. 221–223°. (Found: C, 81·3; H, 5·0; N, 6·4%. $C_{15}H_{11}NO$ requires C, 81·4; H, 5·0; N, 6·3%.)

4-Hydroxy-4′-amidinostilbene hydrochloride (29). This was prepared from 4-hydroxy-4′-cyanostilbene according to the general procedure of Ashley, Barber, Ewins, Newbery & Self [1942]. *4-Hydroxy-4′-amidinostilbene hydrochloride* crystallized from hot water in long yellowish needles, m.p. 316–317° (decomp.). (Found: C, 65·6; H, 5·4; N, 9·9; Cl, 12·5%. $C_{15}H_{15}ON_2Cl$ requires C, 65·6; H, 5·5; N, 10·2; Cl, 12·8%.)

SUMMARY

1. A series of compounds of the stilboestrol type has been prepared and tested *in vitro* for bactericidal activity against staphylococci and streptococci. Several have been found to possess exceptionally high activity, the most active being

$$4\text{-OH}.C_6H_4.CEt.CEt.C_6H_5,$$

which is some 40 times as active as the mould metabolic product citrinin, and as active as chloroxylenol under the conditions employed.

2. A comparison of the bactericidal and oestrogenic activities of these compounds shows that the two actions are not necessarily concurrent, though both probably depend on the ready acceptance by the cell of molecules of the cyclopentanophenanthrene and stilboestrol types.

3. *In vivo* the compounds tested showed little or no activity, possibly on account of their very low solubility.

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Growth-promoting Substances from Bacteriological Filter Pads

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During an investigation of the nutritional requirements of acid-fast saprophytes [Edson & Hunter, 1943], synthetic media were employed, solutions of possible growth stimulants being added aseptically to a basal salt mixture. The methods of sterilization were selected according to the nature and stability of the chemical compounds involved. It soon became evident that the results varied with the mode of preparation and filtration of the test substances. It was shown that solutions, both in water and in saline, were able to extract materials from filter pads in a concentration sufficiently high to stimulate the growth of acid-fast organisms, so that false positive results were sometimes recorded.

MEDIA AND METHODS

The salt mixture, at pH 7·5, was the one described by Edson & Hunter [1943]; the medium and the inoculum were prepared, and growth estimated, as before. Stock solutions of the source of carbon (glucose) and solutions (usually 10 ml. quantities) of substances to be tested were sterilized by filtration through Seitz E.K. or Ford S.B. pads clamped in the glass and steel holders described by Knight & Fildes [1936].

By these methods clear-cut results were obtained, and it was shown that of over 200 different compounds tested, those which were active belonged to four classes: (1) citric acid, (2) α-amino-acids or compounds in which the amino groups were bound in peptide linkages, (3) histamine, (4) ions of calcium and iron.

Growth of Mycobacteria *in media sterilized by different methods*

Anomalous results were first clearly found when solutions of 2:5-diketopiperazine were sterilized by different methods. The results for *Myco. phlei* are set out in Table 1. When growth occurred, it was copious and was checked by microscopic examination of stained smears, and by subculture of a small portion of surface film on Dorset's egg medium.

Autoclaving of aqueous and saline preparations of 2:5-diketopiperazine (flasks 1 and 2) probably induced hydrolysis to glycyl-glycine, which was subsequently shown to be a growth stimulant. It was evident that the anhydride was inactive (flask 5), and that the methods of preparation and sterilization were responsible for the difference in flasks 3 and 4.

Similar examples of 'false' stimulation were encountered when it became necessary to filter the final medium containing other test substances or when the solubility of some compounds precluded the preparation of concentrated stock solutions. Whenever heat-stable substances were added to the medium and autoclaved, no false results were obtained. 'False' stimulation occurred, however, if small quantities of the test substance were added to the basal salt mixture and the whole passed through a 3 or 6 cm. filter pad. Many of the unstable compounds under investigation proved to be sufficiently free from bacterial contamination to permit their use without previous sterilization, and thus it was possible to prove that the stimulation in filtered solutions was 'false'.

Filtration of the basal salt mixture alone through a pad caused stimulation of the growth of acid-fast saprophytes in the presence of a suitable source of carbon. Table 2 shows results of tests in media under different conditions. Growth in a filtered basal medium is compared with true stimulation by citrate and by *dl*-alanine.

The stimulant effect was progressively greater as successive 20 ml. portions of salt mixture were passed through the same pad.

One other organism, *Esch. coli* (laboratory stock strain), was also tested, the same basal salt mixture

Table 1. *Effect of method of sterilization of synthetic medium on growth of* Myco. phlei

	Flask no.				
	1	2	3	4	5
Source and treatment of 2:5-diketopiperazine	0·4 ml. of 0·5% autoclaved stock solution added aseptically to autoclaved salt mixture	2 mg. solid added to salt mixture and autoclaved	0·4 ml. of 0·5% filtered stock solution added aseptically to autoclaved salt mixture	2 mg. solid added to salt mixture and filtered	2 mg. unsterilized solid added to autoclaved salt mixture
Growth at 7 days	+	+	Nil	+	Nil

Medium: 20 ml. basal salt mixture containing 1% of glucose and 0·01% 2:5-diketopiperazine. Inoculum, 0·01 mg. Temp. 38°.

Table 2. *Growth of saprophytic* Mycobacteria *in different media*

Inoculum: 0·01 mg. seeded in 20 ml. medium. Time, 7 days. Temp. 38°.

	Growth (mg. dry wt.) on				
Organism	Autoclaved basal salt mixture +1% glucose	Filtered basal salt mixture +1% glucose	Autoclaved basal salt mixture +autoclaved citrate (0·01%) +1% glucose	Basal salt mixture +*dl*-alanine (0·01%) autoclaved +1% glucose	Broth controls
Myco. phlei	Nil	6·4	9·6	11·8	46·0
Myco. stercoris	Nil	6·7	9·5	8·0	37·1
Myco. smegmatis	Nil	6·2	9·6	13·2	50·2
Myco. sp. *Karlinski*	Nil	6·5	8·0	8·0	33·4
Myco. sp. *leprous Kedrowsky*	Nil	1·4	Trace	Trace	5·8

being employed, with an inoculum of one drop of a slightly opalescent suspension of washed bacilli. The results were read within 48 hr. and were unchanged after 3 days. No growth occurred on inoculation when glucose or citrate was the source of carbon in the autoclaved basal mixture, but the addition of glucose (1 %) and citrate (0·01 %) to autoclaved salt mixture stimulated heavy growth. When the basal salt mixture containing glucose was filtered through a pad, strong stimulation equal to that obtained by citrate addition was observed. The growth-promoting quality of Seitz filtrates was therefore not confined to species of the genus *Mycobacterium*.

The nature of the growth-promoting substances

The active materials were more easily extracted by water or saline mixture when these were hot than when cold and hence the substances were readily extracted by autoclaving portions of a pad in 20 ml. of basal mixture. Ether and alcoholic extracts were inactive, while tests for citric acid in aqueous extracts were consistently negative within the limits of the micro-tests employed. The pads appeared to consist mainly of asbestos and paper pulp; average ash content, 36·0 %. Sodium silicate, tested as a possible stimulant, was ineffective. Although the water-soluble portion of pad ash (completely ashed in an electric furnace) caused little, if any, growth stimulation, the neutralized acid-soluble (HCl Analar) fraction produced a growth averaging 7·6 mg.

The results indicated that the stimulating inorganic material remained after ashing in a form soluble in dilute HCl. Of the numerous inorganic elements tested [Edson & Hunter, 1943], all were ineffective with the exception of Ca and Fe. It was found that Ca^{++} (50 μg.) and Fe^{+++} (20 μg.), produced growth stimulation approximately equal to that given by pad ash.

The filtered basal medium was very slightly turbid, and its behaviour on heating and cooling was suggestive of the presence of calcium phosphate. A positive test was given with ammonium oxalate, indicating, by comparison against known standards, the presence of about 100 μg. Ca^{++}/20 ml. of medium. The Prussian blue test [Feigl, 1937] revealed a trace of Fe^{+++}, 1–10 μg./20 ml., but the α:α'-dipyridyl test for Fe^{++} was negative. Filter pads, extracted with HCl (Analar Fe-free) gave strong positive tests for Fe^{+++} with thiocyanate reagent, and analysis showed the presence of 250 μg./g., or about 730 μg./6 cm. pad. The pads contained only a trace of Fe^{++} (α:α'-dipyridyl). The iron, however, was not soluble to any great extent in the basal salt mixture. Pad ash gave very strong tests for Fe^{+++} (KCNS) and Ca^{++} (oxalate). It is also of interest to note that acid extracts of pads gave positive tests for copper, determinations based on the diethyl-dithio-carbamate colour reaction [Callan & Henderson, 1929] showing the presence of 10–15 μg./6 cm. pad.

Since previous experiments [Edson & Hunter, 1943] had shown that the growth of acid-fast saprophytes was stimulated by low concentrations of Ca and Fe, it appeared that the presence of these ions was responsible for the results obtained with solutions which had been filtered through the pads. It was further shown that the filtered stock 10 % glucose solutions contained Ca^{++} and traces of Fe^{+++}. In practice 2 ml. of 10 % glucose were added to the salt mixture to give 1 % sugar in the final medium. It was found, however, that 20 ml. of salt mixture, containing 2 ml. glucose solution, gave negative tests for Ca^{++} (by the method used), and that small additions did not supply a sufficiently high concentration of calcium ions to stimulate growth during the period of the test. Similarly the aseptic addition of small volumes, usually less than 0·5 ml., of preparations of test substances (as in Table 1) to autoclaved synthetic medium did not convey sufficient material to affect the growth of *Mycobacteria* under the conditions employed.

DISCUSSION

The findings reported show the possibility of unwittingly adding extraneous materials during filtration of media employed in investigations of bacterial nutrition. Ions of calcium and iron, which have been shown to be growth stimulants for members of the genus *Mycobacterium*, were extracted from filter pads. By comparison of the type of growth stimulated by Ca^{++} and Fe^{+++} separately with that promoted by pad ash and extracts, it was evident that calcium ions were the main stimulating agents and that not enough iron was extracted to affect growth. The growth produced with pad materials and by Ca was relatively unpigmented and somewhat slimy in character, while that with Fe was granular and deeply pigmented. It was noted, also, that whenever the pad filtrates were very slightly turbid, i.e. when the calcium phosphate or traces of fibre which the pads yielded were just visible, the growth of *Myco. phlei* was more profuse. This is analogous with the findings of Bigger & Nelson [1941], on the effect of talc and other insoluble inorganic substances on the growth of coliform bacilli in water.

It is not suggested that the results eliminate the presence of organic growth factors in pads, but in view of the fact that the stimulation obtained in filtered media was of the same order and appearance as that given by pad ash, it seems likely that, for the *Mycobacteria* at least, the ions of Ca and possibly of Fe are the principal growth-stimulating substances extractable from ordinary bacteriological filter pads.

SUMMARY

In a study of the nutritional requirements of various species of the genus *Mycobacterium*, Seitz filtration

was employed for the purpose of sterilizing certain preparations of possible growth stimulants. It was found that Ca^{++} and Fe^{+++} were extracted from filter pads in a single filtration. These ions were growth stimulants for the acid-fast organisms under the conditions used, and their presence in filtrates accounted for anomalous results obtained with some preparations.

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The Mechanism of Coprosterol Formation *in vivo*

2. ITS INHIBITION BY SUCCINYL SULPHATHIAZOLE AND BY CARBARSONE

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The discovery that brain contains a substance, probably identical with or allied to the cerebroside phrenosin, which enables the organism to convert large amounts of cholesterol, added to the diet, into coprosterol [Rosenheim & Webster, 1941], offers a convenient experimental method for investigating the effect on coprosterol formation of drugs which act specifically on the intestinal flora or fauna. In view of the surprising lack of evidence for the theory, advanced on purely theoretical considerations by the early investigators [Bondzynski & Humnicki, 1896; Müller, 1900], and held ever since, that putrefactive intestinal bacteria are responsible for the conversion of cholesterol into coprosterol by direct hydrogenation, we selected in the first instance the sulphonamide derivative succinyl sulphathiazole (S.S.) for investigation. This drug exerts a powerful bacteriostatic action on *Escherichia coli* and other coliform bacteria of the intestine and has the additional advantage of low toxicity, owing to its poor absorption from the alimentary tract.

As the outcome of this investigation, together with other experimental evidence, made it improbable that intestinal bacteria have a share in coprosterol formation, the possibility was considered whether the fauna, and especially the protozoan population, of the intestine rather than its flora was concerned in the process. This possibility was investigated by examining the coprosterol excretion of rats treated with the amoebicidal drug carbarsone (*p*-carbamino phenylarsonic acid). This arsenical also frees the intestinal tract from the flagellate *Trichomonas*, a non-pathogenic parasite occurring in the intestinal canal of man and animals. A drug specifically acting on *Trichomonas* was chosen for the test, since it has been claimed that cholesterol is an essential growth factor for four species of *Trichomonas* [Cailleau, 1937; 1939].

EXPERIMENTAL

Methods

The technique of the animal experiments, the composition of the diet, and the analytical methods used have previously been described [Rosenheim & Webster, 1941]. S.S. was administered by intimately mixing the drug with the diet to the amount of 5% [Welch, Mattes & Latven, 1942]. The S.S. contents of the faeces were determined by the method of Bratton & Marshall [1939]. Carbarsone was given in the drinking water, which consisted of a 0·5% solution of the drug in a 1% solution of sodium hydrogen carbonate in distilled water [Gabaldson, 1936]. *Esch. coli* counts were made by the usual method, using poured MacConkey's plates. The faeces and intestinal contents were examined for *Trichomonas* microscopically and by cultivation in the medium of Dobell & Laidlaw [1926]. The protozoological examinations were kindly made by our colleague, Dr Clifford Dobell.

Experiments with succinyl sulphathiazole

In a preliminary experiment two groups each of three male rats were used. During a period of 3 days they were fed on the stock diet, the experimental group receiving S.S. as well. Bacteriological examination of the faeces on the fourth day showed that *Esch. coli* had been almost eliminated in the experimental group, whilst the faeces of the control group contained 50×10^6 organisms/g.

In the subsequent period both groups were kept for 4 days on a diet of steamed brain (15 g./day/rat), the experimental group receiving S.S. as before. The faeces of this group were again found to be free from *Esch. coli*, the control group containing 80×10^6 organisms/g. Both groups lost in weight, the experimental group losing twice as much as the control groups. The analytical results are summarized in Table 1.

Table 1. *Influence of the addition of succinyl sulphathiazole to a diet of brain, on the faecal excretion of coprosterol and of* Esch. coli, *by the rat*

Period	Duration of period (days)	Total wt. of food consumed (g./day)	Total body weight: Initial (g.)	Total body weight: Final (g.)	Total body weight: Loss (g./day)	Total wt. of moist faeces (g.)	Faecal excretion, during last 24 hr. of period, of: *Esch. coli* (no./g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of dry faeces)	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	45	568	509	19·7	40·7	80×10^6	None	None	3·39	2·54	74·9
Experimental	3	45	534	418	38·7	42·7	1×10^3	4·6	13·6	2·34	Trace	<1

The addition of S.S. to the brain diet produced almost complete inhibition of coprosterol formation, whilst the coprosterol excretion of the control group on brain diet alone was of the same order as in our previous experiments [Rosenheim & Webster, 1941]. Pure cholesterol, m.p. 148–149°, was recovered from the digitonides obtained in the estimation of the sterols in the faeces of the experimental group, whilst those of the control group yielded coprosterol, m.p. 100–101°, in typical needles.

The administration of S.S. was stopped on the fifth day and the animals were put on a diet of brain alone, for 4 days. Only one of the rats survived this period, the high mortality rate probably being due to the increased toxicity of the drug when given in a diet low in protein [cf. Smith, Lillie & Stohlman, 1941; Kapnick, Lyons & Stewart, 1942]. Examination of the faeces of the surviving animal showed, however, that in spite of the return, in enormously greater numbers, of coliform bacteria (100×10^6/g.), the coprosterol content amounted to only 7 % of the total sterols.

In the next experiment the high mortality rate was avoided by substituting for brain the standard diet supplemented by cholesterol (160 mg./rat/day) and cholesterol-free brain powder (1·6 g./rat/day). The fact that the specimen of brain powder [Rosenheim & Webster, 1941] used had been prepared 6 years ago and had retained its full activity, incidentally proves the stability of the factor contained in it.

Two groups of three male rats each were used, the experimental group receiving S.S. for a preparatory period of 3 days. The experimental period, with the additional supplement of brain powder and cholesterol + S.S., lasted 4 days, the faeces of the last 3 days being collected for analysis. During this and part of the subsequent period the faeces were semifluid and unavoidable losses occurred in their collection. After 4 days the administration of S.S. was stopped and the animals were kept on the standard diet and brain powder + cholesterol for 7 days.

The results (Table 2) were practically identical with those of the previous experiment, in which brain was the sole food. The animals remained in good health, however, and during the short experimental period they actually increased in weight at the same rate as the control rats. In this connexion it is significant that the S.S. content of the faeces

Table 2. *Influence of the addition of succinyl sulphathiazole to a normal diet supplemented with cholesterol and brain powder, on the faecal excretion of coprosterol and of* Esch. coli, *by the rat*

Period	Duration of period (days)	Rats in group	Total wt. of food consumed (g./day)	Total body weight: Initial (g.)	Total body weight: Final (g.)	Total body weight: Increase (g./day)	Total wt. of moist faeces (g.)	Faecal excretion, during last 24 hr. of period, of: *Esch. coli* (no./g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of dry faeces)	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	3	34	486	505	6·3	13·0	17×10^6	None	None	1·02	0·77	75·5
Experimental	3	3	31	463	480	5·7	16·6	1×10^3	7·5	27·7	0·62	Trace	<1
Post-experimental	6	3	33	480	518	6·3	16·1	32×10^6	None	None	1·05	Trace	<1

on the last day of the experimental period was nearly double that of the faeces on the corresponding day in the preceding experiment (see Table 1), in which brain was the exclusive diet. A greater absorption of the drug from the intestinal tract may thus explain the higher mortality on the brain diet.

The persistence of the inhibitory effect on coprosterol formation was again shown, in spite of the return of *Esch. coli* in the faeces after the withdrawal of the drug. The duration of this inhibitory period has not yet been studied in detail, but in one experiment it was found that the coprosterol excretion was still *c.* 15 % below the normal level after 3 weeks on the standard diet.

Experiments with carbarsone

In a preliminary experiment a male rat, weighing 220 g., was taken at random from the Institute's colony. On microscopical examination of its faeces, the animal was found to be heavily infected with *Trichomonas muris*. A feeding test with steamed sheep's brain showed a coprosterol excretion of 78 % of total sterols. The rat then received 45 mg./day of carbarsone whilst on the standard diet for 2 days. During the next 5 days the standard diet was replaced by steamed sheep's brain (25 g.) containing bone meal (1 g.) and the dosing with carbarsone was continued. The rat received 210 mg. of carbarsone, i.e. 42 mg./day. At the end of this period the faeces were free from *Trichomonas*, but showed a high count of *Esch. coli* (100×10^6/g.). The faeces of the last 4 days were collected and analyzed. The sterols consisted of practically pure cholesterol containing only traces of coprosterol.

This striking inhibitory effect of carbarsone on coprosterol formation was confirmed by the subsequent experiments, which were carried out on individual animals in order to ensure adequate control in the collection of faeces for bacterial and protozoological examination. Male rats of the average weight of 270 g. were kept in separate cages with coarse wire-grid bottoms. For the first 5 days their diet consisted of a mixture, in equal parts, of the standard diet and steamed sheep's brain. All the rats were found to be infected with *Trichomonas* and bacteriological examination of the faeces showed the usual high count of *Esch. coli* at the end of this period. During the carbarsone period the drug was administered for 1 day in the drinking water, whilst the rats were on the standard diet. During the following 5 days they received known amounts of carbarsone (see Table 3) in their food, consisting of the mixture of steamed brain and standard diet described above. At the end of the experimental period the rats were in good condition. The faeces were found to be free from *Trichomonas*, whilst the *Esch. coli* counts were as high as before. The results of four experiments of this type, in which the faeces of the last 3 days were collected and analyzed, are given in Table 3.

The figures in the last column of Table 3 demonstrate conclusively the inhibitory effect of carbarsone on coprosterol excretion, which has been reduced to the merest traces even in the rat in which, before the administration of carbarsone, coprosterol accounted for as much as 90 % of the total sterols excreted.

Attempted bacterial hydrogenation of cholesterol and cholestenone

The possibility that the failure of previous attempts to effect coprosterol formation by intestinal bacteria *in vitro* may have been due to the absence in the media used of an essential growth factor, led us to investigate their action on cholesterol and cholestenone in the presence of brain, which favours coprosterol formation *in vivo*.

(1) *Cholesterol.* (*a*) Flasks containing minced sheep's brain (10 g.) inoculated with faeces (1 g.) from rats fed on brain, were incubated in anaerobic jars at 37° for 8 and 16 days respectively. The unsaponifiable matter was prepared in the usual

Table 3. *Influence of the addition of carbarsone to a normal diet supplemented with brain, on the excretion of coprosterol by the rat*

Period	Duration of period (days)	Rat no.	Carbarsone intake (mg./day)	Total wt. of moist faeces (g.)	*Trichomonas* infection	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	1	0	10·0	+ + +	0·83	0·62	74·7
	3	2	0	6·2	+ + +	0·81	0·68	83·9
	3	3	0	7·7	+ + +	0·82	0·74	90·3
	3	4	0	6·1	+ + +	0·65	0·52	80·0
							Average	82·2
Experimental	3	1	69	10·5	None	0·73	Trace	<1
	3	2	64	8·4	None	0·69	Trace	<1
	3	3	63	12·1	None	0·88	Trace	<1
	3	4	60	7·6	—*	0·64	Trace	<1

* Not examined.

way for the estimation of total and saturated sterols by the gravimetric digitonin method. The cholesterol content of the brain was 2·6 % and the mixture in the control flask contained 37 mg. of coprosterol, derived from the added faeces. At the end of the experiment 36 mg. (± 7) of coprosterol were found in the incubated mixtures, showing that no conversion of cholesterol into coprosterol had taken place. (*b*) In another set of experiments 10 g. portions of minced sheep's brain, ground up with 20 ml. of glucose broth, were sterilized and inoculated with 5 ml. of a 24-hr. culture of mixed anaerobic bacteria. This culture was obtained from the caecal contents of three rats excreting large quantities of coprosterol on a diet of sheep's brain. After 17 days' incubation under anaerobic conditions the brain mixtures yielded 2·55 % and the control 2·65 % of cholesterol. No coprosterol had been produced.

(2) *Cholestenone.* (*a*) Suspensions of finely powdered cholestenone (200 mg.) in glucose broth (50 ml.) were sterilized and inoculated with a fresh mixed culture of intestinal bacteria (see above). No sterols precipitable by digitonin were obtained from these mixtures after anaerobic incubation for 17 days. It has previously been shown [Mamoli, Koch & Teschen, 1939] that cholestenone was not attacked by a pure culture of *B. putrificus* under the conditions in which the keto group and ethenoid linkage of testosterone, androstenedione, etc. were reduced. (*b*) Flasks containing 10 g. portions of sheep's brain, inoculated with faeces as above, and intimately mixed with a solution of 0·2 g. cholestenone in cetyl alcohol, were incubated anaerobically for 8 and 16 days respectively. The average amount of coprosterol recovered, 33 mg. (± 1), was slightly less than the amount (37 mg.) derived from the added faeces. These negative results agree with those of similar experiments by Diels [1937].

DISCUSSION

It is tempting to associate the striking inhibitory effect of succinyl sulphathiazole on coprosterol formation with the simultaneous disappearance, caused by the drug, of *Esch. coli* and coliform bacteria from the intestine. The conclusion that these bacteria are therefore directly responsible for coprosterol formation is, however, fallacious and disproved by the fact that the inhibitory effect persists in spite of the immediate return, in enormously increased numbers, of *Esch. coli* and coliform bacteria after the withdrawal of the drug.

The concept that coprosterol originates in the intestine by bacterial hydrogenation of cholesterol arose at a time when the stereochemical differences in the constitution of the reduction products of cholesterol were entirely unknown. Since then it has been recognized that, under all known conditions, the hydrogenation of the Δ^5 ethenoid linkage of cholesterol leads to the *trans*-decalin derivative (β)-cholestanol and that coprosterol, the corresponding *cis*-decalin derivative, can be obtained only by indirect methods. In ignorance of these facts, the early investigators linked up coprosterol formation in the intestine with the activity of 'putrefactive' bacteria, an apparently obvious interpretation of the facts known to them. Although this simple solution of a complex problem has not been supported by any convincing experimental evidence, it is still current. As an argument against the bacterial origin of coprosterol the fact may be adduced that bacteria are devoid of cholesterol, a sterol found in animal cells only. Moreover, bacteria and the primitive unicellular algae, Myxophyceae [Carter, Heilbron & Lythgoe, 1939], are the only cellular organisms in which sterols of any kind are absent.

Evidence is accumulating in favour of the view that the chemical changes occurring in coprosterol formation proceed in two stages, consisting in a primary oxidation of cholesterol to cholestenone followed by reduction of the latter to the *cis*-decalin derivative coprosterol [Rosenheim & Webster, 1943]. Whether these reactions are brought about by the enzymic activity of the intestinal mucosa or by other agencies is unknown. Our results show that intestinal bacteria in any case are unable to reduce cholestenone to coprosterol *in vitro*, even in the presence of the hypothetical growth factors, contained in brain, which favour coprosterol formation *in vivo*. In this connexion the recent work on the bacterial reduction and oxidation of steroid sex hormones, carried out mainly in Butenandt's laboratory, acquires particular importance. Some of the incidental observations recorded in these researches have a direct bearing on the coprosterol problem, since they led to the conclusion that the susceptibility of steroids to bacterial attack is dependent on the length of the side chain: cholesterol, cholestenone and cholestanone, possessing the intact side chain of eight carbon atoms, are recovered unchanged when subjected to the action of certain dehydrogenating or hydrogenating bacteria respectively, under the same conditions which bring about the oxidation or reduction of the functional groupings of steroid sex hormones possessing a shorter or no side chain [Mamoli & Schramm, 1938; Mamoli, 1938; Mamoli *et al.* 1939]. It is therefore not surprising that all attempts to convert cholesterol into coprosterol by bacterial action *in vitro* should have failed, and it appears unlikely that cholesterol is concerned in bacterial metabolism.

If we accept the view that bacteria have no share in coprosterol formation, it seems attractive to consider as an alternative the possible role of the intestinal fauna, and especially of protozoa, in this

process. Various species of flagellates belonging to the genus *Trichomonas* are non-pathogenic inhabitants of the intestinal tract of man and animals, so that the caecums and intestines of rats 'are often swarming with *Trichomonas*' [Wenyon, 1926]. A possible connexion between these protozoa and cholesterol metabolism is suggested not only by the fact that four species of *Trichomonas* require cholesterol as an essential growth factor [Cailleau, 1937; 1939], but also by various other considerations. Thus, infection with *Trichomonas* of babies up to 1 year is rare [Faust & Headlee, 1936] and their faeces, like the meconium, are devoid of coprosterol [Flint, 1862; Müller, 1900; Fox & Gardner, 1925]. When the consumption of a mixed diet at later stages of their development favours the chances of infection by the flagellates, coprosterol appears in their excreta. Further, an exclusive milk or meat diet reduces both the trichomonad infection [Hegner, 1923; 1924] and coprosterol excretion [Müller, 1900; Dorée & Gardner, 1908].

Attractive as this suggested correlation between protozoa and coprosterol formation seemed to be, and in spite of its apparent confirmation by the results of the above-described experiments with the arsenical drug carbarsone, such a correlation appeared to be fortuitous. On further investigation, it was found that rats, freed from *Trichomonas* by carbarsone treatment, excreted a considerable amount of coprosterol in the period following the stoppage of the drug. On the other hand, administration of succinyl sulphathiazole caused the evacuation of large numbers of *Trichomonas* in the faeces without freeing the rats from the infection, but they excreted only small amounts of coprosterol for some time after cessation of the treatment. It is realized that our exploratory experiments in this direction are liable to various interpretations. The results so far obtained, however, indicate that the participation of protozoa in coprosterol formation is at least as unlikely as that of bacteria.

It is equally difficult to relate the interference with cholesterol metabolism exhibited by carbarsone and succinyl sulphathiazole with the inhibitory effect of the latter drug on the synthesis of the essential vitamins, biotin and folic acid, by intestinal bacteria [Nielsen & Elvehjem, 1942; Welsh & Wright, 1943], especially as a possibly similar effect of carbarsone has not yet been investigated.

The unexpected fact thus emerges that two drugs of dissimilar chemical constitution, one of which acts specifically on *Esch. coli* and not on protozoa and the other on Protozoa but not on *Esch. coli*, have in common an inhibitory effect on coprosterol formation, which does not seem to be due to their direct action on either the bacteria or protozoa concerned. The mechanism of this inhibitory action remains so far unexplained.

SUMMARY

1. The bacteriostatic action of succinyl sulphathiazole on intestinal coliform bacteria was found to be concurrent with a complete inhibition of coprosterol formation. The apparent correlation, however, between *Esch. coli* and coprosterol formation is fortuitous and the available evidence makes it improbable that intestinal bacteria have any share in the process.

2. The possibility that the fauna, and especially the protozoan population, of the intestine rather than its flora is concerned in coprosterol formation was investigated by examining the coprosterol excretion of rats treated with carbarsone (*p*-carbamino phenylarsonic acid). This amoebicidal drug frees rats from *Trichomonas muris* infection and was found also completely to inhibit coprosterol formation. Exploratory experiments, however, indicate the absence of a relationship between protozoa and coprosterol formation.

3. The complexity of the problem is made apparent by this interference with cholesterol metabolism, shared by an antibacterial and an amoebicidal drug, which seems to be unconnected with their action on either bacteria or protozoa and which remains so far unexplained.

We are indebted to Dr Clifford Dobell, F.R.S., for making the protozoological examinations, to Dr A. T. Fuller for colorimetric estimations of succinyl sulphathiazole and to Messrs W. T. Milton and F. A. Holbrook for bacterial counts. Our thanks are due to Dr A. J. Ewins, F.R.S., of May and Baker, Ltd., for a gift of succinyl sulphathiazole.

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A Micro-method for the Estimation of Vitamin B_1

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A micro-method for the estimation of vitamin B_1 has been described by Atkin, Schultz & Frey [1939]. It is based on the measurement of the rate of fermentation of sugar by baker's yeast, using a Warburg or similar apparatus, and is sensitive to 0·005 μg. of vitamin B_1. The method described below is based on the thiochrome technique and is of similar sensitivity. It was developed during work on the distribution of vitamin B_1 in the wheat grain and has been applied successfully to small quantities (1–50 mg.) of cereal products [Hinton, 1942].

EXPERIMENTAL

The method is based upon the optical design of the Spekker Photoelectric Fluorimeter. In this instrument, an image of the mercury discharge tube is brought to a focus at a point opposite the photo-cell, the focused beam normally passing through the test solution contained in a large glass cell. In the micro-method here described, the glass cell is replaced by a suitable holder by means of which a capillary tube containing the test solution is placed in the image, which measures 2 × 20 mm. approximately in the focal plane, and thus receives the full irradiation transmitted by the system (Fig. 1).

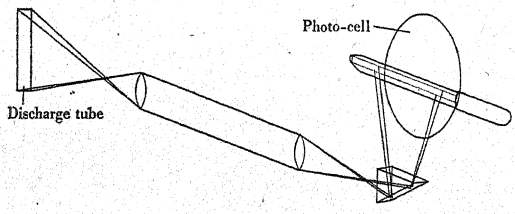

Fig. 1. Diagram showing optical design of the Spekker Photoelectric Fluorimeter and position of capillary-tube test cell used in the micro-method.

The Spekker Fluorimeter was used in conjunction with a Cambridge Short-Period Galvanometer of sensitivity 3000 mm./μA., and resistance 196 ohms, which was used at full sensitivity.

Procedure

The strength of ferricyanide to produce maximum fluorescence in the test solution is first ascertained by the procedure summarized below. With our cereal extracts this was found by testing a range of strengths from 0·05 to 0·5 %, in steps of 0·05 %. For standard aneurin solutions the best concentration was 0·005 %.

The pipette to be used for the test solution is first washed out with the solution, in readiness. 0·1 ml. of 30 % NaOH is measured into a 2 × $\frac{3}{8}$ in. tube, 0·02 ml. of the appropriate ferricyanide solution run in on top and left undisturbed. 0·1 ml. of the test solution is then measured out and, immediately before this is added to the tube, the NaOH and ferricyanide are mixed by shaking. The test solution is run in and the whole again mixed by shaking. Oxidation is carried out in this way with each tube in a series which, in order to check the calibration curve, includes three standard aneurin solutions of different strengths. 0·2 ml. of water-saturated isobutanol is then added to each tube, and extraction carried out by stirring for 30 sec. The tubes are allowed to stand for 10–15 min. to allow separation of the layers, those containing cereal extracts being centrifuged for 3–4 min. at 1500 r.p.m. to improve the separation. The isobutanol layer is drawn up directly into the capillary tube for measurement in the fluorimeter. A no. 39 Wratten filter was used in front of the right-hand photo-cell (Fig. 1).

The calibration curve was prepared by the same procedure, using solutions containing from 0·001–0·2 μg. aneurin/0·1 ml.

Examination of the procedure

Accuracy of the method. Standard aneurin solutions in $N/20$ HCl were found to be stable until cloudiness and growth developed. In most cases they were stable for several months. Cereal extracts were made with $N/5$ HCl [Nicholls, Booth, Kent-Jones, Amos & Ward, 1942] applied on a micro scale.

In the case of solutions of pure aneurin the extreme variations between replicates were $\pm 20\%$ at concentration of aneurin in the region of $0{\cdot}001\,\mu g.$, decreasing to $\pm 3\%$ at $0{\cdot}05\,\mu g.$

It should be noted that attention was paid to the possibility of decomposition of thiochrome by light during the preparation of the thiochrome solution, especially in the case of the smallest quantities. Readings obtained when the operations were carried out near a window in the full daylight of a clouded sky did not, however, differ significantly from those carried out in very weak artificial light (1–2 candle power). The only precaution taken, therefore, was to shade the operations from sunlight or from very bright sky light.

The capillary test-cell and holder. Greatest sensitivity was obtained with a glass tube of oval section and 1×3 mm. bore. For good results certain details required attention: the holder (Fig. 2) was made so that the angle which the

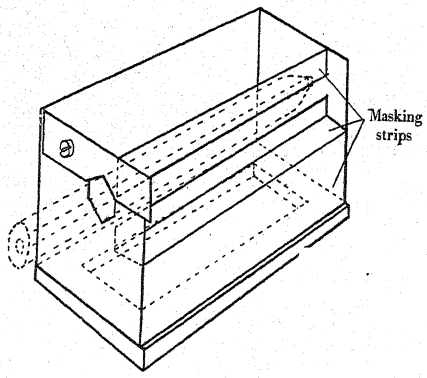

Fig. 2. Holder made from sheet metal with $\frac{1}{8}''$ brass base to fit the cell carrier of the Spekker Photoelectric Fluorimeter and hold the capillary-tube test cell at the correct angle and position.

oval section made with the light beam could be adjusted to the optimum and the tube adjusted squarely in the beam; fluorescence from the glass wall was masked off; adjustments were made to the lamp-house, sliding carrier, etc. of the fluorimeter to make the whole as stable as possible; additional shielding of the photo-cells was provided and great changes in illumination of them during a period of use was avoided.

The length of the tube was 8 cm. To fill it a rubber 'policeman', slipped right down over the end of the tube, is slid carefully upwards whilst the point dips below the surface. The contents are ejected by the reverse movement. On each occasion before the tube is placed in position for measurement the outside should be cleaned by moistening with ethanol and polishing with a cloth. A considerable amount of isobutanol remains on the inside of the tube after it is emptied and the tube should therefore be rinsed with two lots of the solution to be measured. This is done by drawing up a drop of the solution and running it along the tube. The same tube was used for measurement of blank, standard and unknown, and it should be marked and always placed in the holder the same way up.

Standard of fluorescence. As a permanent standard, a crystal of fluorite was used, which was mounted in a holder and masked by a diaphragm to give a fluorescence intensity of the same order as a solution of quinine sulphate of $0{\cdot}5\,\mu g./ml.$

Expression of results. The figures given in the text to illustrate each point are expressed in a relative form. They were expressed in terms of aneurin found on the basis of the fluorescence reading, and then recalculated for each series, the highest yield in the series being taken as 100.

Optimum volumes of reagents. To attain maximum sensitivity, the amount of isobutanol was kept at a minimum. The tube required 0·12 ml., and a total of 0·2 ml. provided enough excess to give the necessary two rinsings. Isobutanol saturated with water was used. 0·1 ml. was chosen as a convenient quantity of test solution, and 0·1 ml. of NaOH gave a total volume most suitable for the subsequent extraction. The smallest quantity of potassium ferricyanide solution which could be handled with confidence is 0·02 ml., and this amount was used in all cases. It was found most satisfactory to keep the volume constant in each case and to adjust the concentration of the solutions as necessary. The reactions were carried out in test-tubes $2 \times \frac{3}{8}$ in.

Handling of these small volumes was facilitated by constructing special pipettes to hold the quantities required, the capillary tube being drawn off slightly and the tip bent round and ground (Fig. 3). The solutions are blown out on to the side of the tube and can be placed just where required.

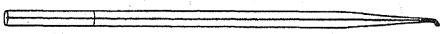

Fig. 3. Micro-pipette.

Methanol was omitted, as, under these conditions, it seriously affected the partition of the thiochrome in the two phases. Jansen [1936] used methanol because of its protective action towards thiochrome, which is thus made less sensitive to excess ferricyanide. The methanol, however, increases the proportion of thiochrome remaining in the aqueous phase; this effect is not serious when the volume of isobutanol is many times greater than the aqueous phase. In the present case, the volumes of the two phases are equal and the serious effect of the methanol on the final fluorescence is seen from the following figures:

Methanol (ml.)	Nil	0·01	0·04	0·08
Reading	100·0	95·0	81·5	54·0

These results were obtained by carrying out the oxidation in the absence of methanol, which was added finally with the isobutanol. 0·08 ml., which corresponds to about 30% of the reaction mixture, causes an excessive reduction in sensitivity. The aim was to attain maximum sensitivity, but some reduction could be accepted if tolerance of the thiochrome towards excess ferricyanide was thereby obtained. (Tolerance in this paper is defined as the range of concentration of ferricyanide over which the same fluorescence reading is obtained for a fixed amount of aneurin.) The following figures illustrate the tolerance obtained, with $0{\cdot}2\,\mu g.$ standard aneurin solutions, by the addition to the

reaction mixture of 0·02 ml. of methanol, which produces a permissible drop in sensitivity:

Strength of ferricyanide (%)	0·003	0·006	0·012
Reading	89·0	100·0	100·0
Strength of ferricyanide (%)	0·025	0·05	0·1
Reading	100·0	97·0	93·5

There is a tolerance range of about 8 times. Without methanol, the tolerance range is reduced to twice:

Strength of ferricyanide (%)	0·0006	0·0012	0·0025	0·005	0·01
Reading	47·5	82·0	99·0	100·0	95·0

As 50 times more ferricyanide is required for a cereal extract than for the standard aneurin solutions, the slight increase in tolerance noted above was not worth considering. In Jansen's experiments he used about 60% methanol in the reaction mixture and reported a range of 500 times.

It is noted from the two series of figures that the optimum strength of ferricyanide is 10 times greater when methanol is present. This is because the methanol enters into the reaction, inactivating the ferricyanide. With 0·1% ferricyanide the following figures were obtained:

Methanol (ml.)	0·01	0·04	0·08
Reading	100·0	88·7	6·8

This effect is much greater than the effect on the thiochrome partition. With 0·5% ferricyanide solution, the figures were:

Methanol (ml.)	0·01	0·04	0·08
Reading	89·5	100·0	77·0

In light of the effect of the methanol on partition, this indicates a reversed effect which is due to reduction of the ferricyanide concentration towards the optimum by the action of the methanol.

The inactivation of the ferricyanide proceeds slowly, and the above results were obtained by mixing the NaOH, ferricyanide and methanol together and allowing the mixture to stand some minutes before adding the aneurin solution. The time effect is illustrated by the data obtained with 0·05% ferricyanide:

	Reading
Aneurin (0·2μg.) added immediately on mixing	100·0
Aneurin (0·2μg.) added after 1 hr.	50·0

Aneurin (0·15μg.) added after time intervals as shown:

Min.	3	8	13	18	23	28
Reading	100·0	98·5	96·8	91·5	88·0	78·0

Again, if a stronger ferricyanide solution is used, the effect is first to increase the fluorescence, which is due to reduction in ferricyanide towards the optimum. The following data were obtained with 0·1% ferricyanide:

	Reading
Aneurin (0·2μg.) added immediately on mixing	89·5
Aneurin (0·2μg.) added after 45 min.	100·0

The strength of the NaOH solution has an effect on the speed of this reaction, e.g. with 0·1% ferricyanide and 0·04 ml. methanol, and after the same time of standing these figures were obtained with 0·15μg. aneurin:

NaOH (%)	30	40	60
Reading	100·0	54·0	10·7

Again, with a stronger solution of ferricyanide and the same amount of methanol, the effect is reversed:

Reading with 0·5% ferricyanide	76·0	89·0	100·0

Optimum strength of ferricyanide. Since it is not practicable to add sufficient methanol to affect the tolerance appreciably, the strength of ferricyanide needs adjusting for each type of material tested, to obtain maximum fluorescence. For standard aneurin solutions the optimum strength is 0·005%.

Aneurin (μg.)	Ferricyanide (%) 0·0005	0·001	0·002	0·003	0·004	0·005
0·15	66·3	77·8	91·2	95·5	97·5	100·0
0·01	—	61·6	96·0	86·0	—	100·0
0·005	—	80·0	—	75·0	—	100·0

Aneurin (μg.)	Ferricyanide (%) 0·006	0·007	0·008	0·009	0·01
0·15	97·0	95·0	94·5	93·5	88·0
0·01	—	—	—	—	—
0·005	—	90·0	—	—	—

The optimum strength for various cereal extracts is shown below:

	Ferricyanide (%) 0·05	0·10	0·15	0·20
No. 1 extract	63·0	90·5	100·0	97·0
No. 2 extract	59·0	88·5	99·5	100·0
No. 3 extract	41·2	67·0	91·0	94·0

	Ferricyanide (%) 0·25	0·30	0·35	0·40
No. 1 extract	96·0	94·5	93·0	92·5
No. 2 extract	99·5	96·5	89·0	91·5
No. 3 extract	98·5	100·0	99·0	99·5

A ferricyanide solution of 0·005% is unstable if exposed to strong light, decomposing in 30 min. or so. Protected from the light, it has been kept for 1 week and may be stable for longer. It was preferred to make a fresh solution by diluting a stock solution of 0·5% each day, and to keep it shaded from daylight.

Optimum strength of NaOH. Solutions varying from 7 to 60% have been tested with varying strengths of ferricyanide. The following figures illustrate the results and indicate that 30% is the most satisfactory:

NaOH (%)	20	25	30	35	40
Reading	89·0	96·2	100·0	96·2	89·0

Order of mixing the reagents. The following figures indicate the degree of destruction of 0·2μg. of aneurin when it is mixed with the NaOH and allowed to stand varying times before the ferricyanide is added:

Ferricyanide added after (min.)	0	3	6	9	12	15
Reading	100·0	70·0	47·5	34·0	25·4	14·3

Jansen mixed the aneurin solution and ferricyanide first, adding the NaOH immediately afterwards. Greater fluorescence is obtained, however, by adding the aneurin solution to the mixture of NaOH and ferricyanide:

0·2μg. of aneurin added to ferricyanide	83·5	81·3	77·2
0·2μg. of aneurin added to mixture of NaOH and ferricyanide	100·0	100·0	100·0

The mixture with NaOH is unstable, when the ferricyanide concentration is of the order of 0·005%. The following method of mixing the reagents was therefore adopted: 0·1 ml. of NaOH is placed first in the bottom of the tube. 0·02 ml. of ferricyanide is then pipetted on to the side of the tube just above the surface of the NaOH, so that it runs down and floats on the surface of the solution. This partition is not disturbed until the test solution is measured out and is ready for adding. With the pipette in one hand, the NaOH and ferricyanide are mixed by shaking the tube, the test solution immediately run in and the whole shaken again. All the reagents are thus mixed together within 30 sec. The times shown in the results below are times elapsing between floating the ferricyanide on the NaOH, and mixing with addition of aneurin solution:

Time elapsing (sec.)	20	120	180	240
Reading	99·7	99·7	98·0	100·0

Once this stage has been reached, the addition of, and extraction with, isobutanol can be carried out within any reasonable time. In the following series the stated times elapsed between carrying out the oxidation and extraction with isobutanol.

Time elapsing (min.)	0	5	10	15	20	25
Reading	95·3	100·0	95·3	95·5	95·3	99·0

Extraction with isobutanol. With these small volumes, bubbling a gas through the liquid is not a successful method of mixing the two phases. Stirring was found to be quite satisfactory. A small glass stirrer made from glass rod drawn out to a diameter of about 1 mm. and shaped into an elongated loop 1 cm. long was fastened directly in the chuck of a small laboratory motor. A frictional brake gave the smooth speed control necessary. With standard aneurin solutions, high speeds, 1000 r.p.m. or more, were necessary to break up the phases completely, but with cereal extracts much slower speeds, 200 or 300 r.p.m., were sufficient and essential, since high speeds produced a fine emulsion which could not be broken down by simple methods. Extracts from some products, e.g. wheat germ, emulsified more readily than others, e.g. endosperm; the lowest speed which sufficed to break up the phases was never exceeded. The following experiment, in which the solution was oxidized in bulk and portions taken for extraction, shows that time of mixing is not critical:

Time of mixing (sec.)	5	10	20	40	60
Reading	95·7	99·5	97·5	100·0	99·0

As a measure of safety, 30 sec. was adopted as standard. The two layers are left in the original tubes, the isobutanol being drawn off directly into the capillary test cell. With cereal extracts the isobutanol layer was cleared by centrifuging at about 1500 r.p.m.; this was not necessary with standard aneurin solutions. Within reason, the time elapsing between extraction and measurement in the fluorimeter is not important:

Measured after (min.)	0	5	10	15	20	25
Reading	95·0	97·5	99·0	99·0	100·0	97·0

Calibration curves. Calibration curves have been prepared with different filters in front of the right-hand photocell; three of these are reproduced in Fig. 4. The shape of the curve is dependent upon the transmission of the filter and the quality of the light emitted by the fluorescent standard. With Wratten filter no. 47 (curve not given) the maximum transmission is in the region a little above the thiochrome fluorescence. For this reason, the photo-cell will not respond much below 0·005 μg. The Hilger no. 6, which has a similar but broader transmission, produces a similar shaped curve, *A* in Fig. 4, and as it is much less

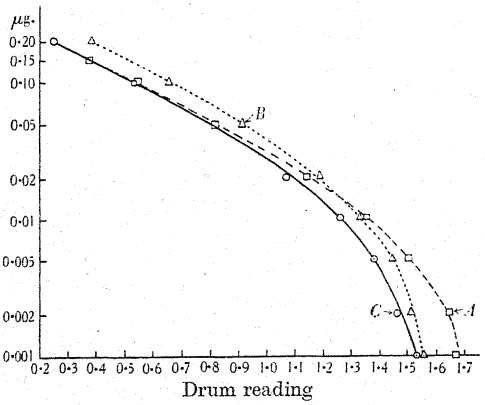

Fig. 4. Calibration curves showing drum reading against micrograms of aneurin measured with: *A*, Hilger filter no. 6; *B*, Wratten filter no. 39; *C*, no filter.

dense, allows the photo-cell to respond to 0·001 μg. The lowest measurements, however, come on an insensitive part of the drum, and with this filter a group of calibration curves shows considerable spread below 0·01 μg. The Wratten no. 39, though of overall density greater even than that of no. 47, transmits well in the region of thiochrome fluorescence and allows response at 0·001 μg. The shape of the curve, *B* in the figure, is closely similar to the shape of the unfiltered curve *C*. This filter also shifts the lowest reading slightly on the drum and a group of curves showed no serious spread over the whole range. On these grounds, no. 39 was judged to be the most suitable filter so far used.

Each curve shown in Fig. 4 is the average of a group of six to eight complete curves. It is desirable to deal with groups of curves in this way as, in common with other photo-electric colorimeters, the Spekker may exhibit a slight shift in the whole curve from day to day. This is particularly the case when it is used as described here, and for this reason it is advisable to put one or two aneurin standards in with each run of estimations. When the capillary test cell containing the thiochrome solution has been placed in position, the photo-cells must be balanced without delay, as the thiochrome is then unstable and the readings fall continuously, especially with solutions of 0·1 μg. of aneurin and above. There is ample time, however, to carry out the balancing precisely, as no significant change in the reading occurs in 30 sec.

SUMMARY

1. A micro-technique for the estimation of vitamin B_1 has been developed. The method is based on the determination of thiochrome with the Spekker Fluorimeter. A special cell is used and a precise plan must be followed with the different

reagents normally used in the conversion to thiochrome.

2. In solutions of pure aneurin (or in cereal extracts) the method is accurate to ± 20 % at concentrations in the region of 0·001 μg., the accuracy increasing to ± 3 % at 0·05 μg.

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Application of the Millon Reaction to the Determination of Chlorophenols in Body Fluids and Tissues

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The versatile antibacterial usefulness of halogenated phenols makes valuable a specific micro-method for their determination in body fluids and tissues [Zondek, 1942]. The present paper describes a semi-micro-method for *p*-chloro-*m*-xylenol (referred to as *CX*) and *p*-chloro-*m*-cresol (referred to as *CC*) in the presence of phenol, e.g. in urine. Patients given *CX* may excrete this substance in concentrations up to 50 mg./100 ml. The halogenated phenol is allowed to react with Millon reagent at room temperature, the coloured substance so formed being extracted with ether and thus obtained in crystal-clear solution suitable for photometric measurement. Phenol itself under these conditions gives a coloured substance which is only sparingly soluble in ether. The method is therefore highly specific.

Several investigators have used the Millon reaction for the determination of tyrosine, e.g. Folin & Ciocalteu [1927]. The present successful extension of such a method to at least two selected chlorophenols suggests that further investigation would render possible a more general application of the Millon reaction to analytical methods, in which the conventional procedures for the estimation of phenols are often inadequate.

EXPERIMENTAL

Reagents. Reagent 1: A mixture of 50 ml. of a 20 % solution of mercuric acetate and 30 ml. of 25 % (v/v) HNO_3 ($d. = 1{\cdot}42$).

Reagent 2: A 1 : 4 diluted solution of 100 g. of mercury in 200 g. of HNO_3 (Millon reagent).

Standard solutions: 1 g. of *CX* or *CC*, twice recrystallized from ethanol and dried *in vacuo*, is dissolved in 5 ml. N NaOH and diluted with H_2O to 1 l. In amber bottles the solutions are constant for at least 1 month.

A. *Procedure with aqueous solutions*

Determination of CX. To 15 ml. of test solution in a test-tube of c. 30 ml. capacity are added 2 ml. of reagent 1 and then, if no precipitate has formed, 1 ml. of reagent 2. If a precipitate forms, filter and carry out the test on a measured portion. The mixture is kept at room temperature for 30 min., cooled in ice-water, and 5 ml. of chilled ether added. The colour is brought into the ether phase by shaking, and the test-tube immediately stoppered. Within the next hour the ether extract is transferred to a 0·5 cm. cuvette covered with a glass slide. The extinction value (*E*) is determined in a Pulfrich photometer with filter S 43. The concentration of the unknown is read by reference to a standard curve prepared by plotting *E* for known solutions similarly treated. This curve should be renewed for each fresh batch of reagent and should be checked every 2 weeks.

Determination of CC. *CC* is determined by a somewhat modified procedure. 1 ml. of reagent 1 and 1 ml. of reagent 2 are added to 10 ml. of the test solution. The mixture is incubated for 2 hr. at 37°, cooled in ice-water for 5 min., and shaken with 5 ml. of cold ether. Photometry is carried out within 1 hr. (filter S 50 and 0·5 cm. cuvette).

Results. The colour formed with *CX* is dirty red in the water phase, but clear yellow in the ethereal solution. With *CC* the colour is red in water and in ether. Typical standard curves are given in Fig. 1. Within a wide range the relation between *E* and the concentration of the halogenated phenol is nearly linear. Concentrations of *CX* or *CC* greater than 0·5 mg./100 ml. were determined with an error of 5 %. Lower concentrations may be determined

by concentrating *CX* or *CC* by extraction from acid solution with a one-third volume of toluene and transferring to a suitable volume of 0·1N NaOH, which is then neutralized with HNO_3. The recovery is practically quantitative. The presence of 0·1N $NaNO_3$ does not affect the test.

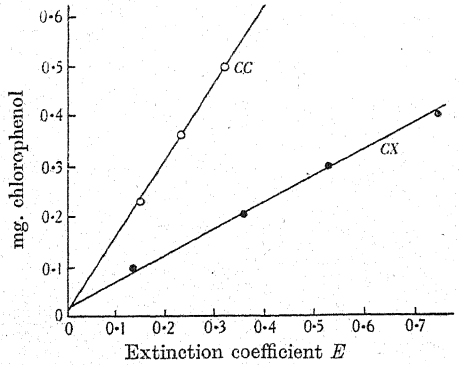

Fig. 1. Dependence of the amount of colour developed with Millon reagent on the quantity of chlorophenol employed. *CC* = *p*-chloro-*m*-cresol; *CX* = *p*-chloro-*m*-xylenol.

Discussion of procedure. By transference from the water phase into ether the coloured substance is concentrated, and at the same time clear solutions suitable for colorimetry are obtained. With *CX* the transfer is quantitative. In the case of *CC* a small fraction is retained in the water phase, but since the distribution ratio between water and ether is constant within the reported range, the value of *E* in ether remains proportional to the *CC* concentration of the original solution (Fig. 1). Furthermore, ether extraction renders the determination relatively specific, the red colour formed by phenol with the reagent being very largely ether-insoluble (see also Table 2).

The inclusion of reagent 1 in the reaction mixtures establishes the desired acidity and accelerates the colour reactions. With solutions containing substances precipitable by Millon solution, reagent 1 also serves to remove the interfering substances. In this case the test solution is centrifuged or filtered 1 min. after addition of reagent 1, and reagent 2 is then added to a portion of the cleared solution (see method for urine). Large variations in the concentration of reagent 1 (one-half to twice the indicated amount) are without effect on the intensity of the colour developed with *CX*. Therefore, the partial removal of reagent 1 by precipitation does not perceptibly affect the colour intensity obtained. In pure aqueous solutions, use of reagent 1 is, of course, not strictly essential.

Under the conditions specified, the indicated reaction times suffice to allow the colour intensity to reach a peak. Longer incubation periods are inconvenient and may entail some fading of the colour. Attempts to hasten the reaction by the use of higher temperatures were unavailing. At 60° the values of *E* were relatively low; at 95° *CX* failed altogether to give a colour with Millon reagent. On the other hand, non-halogenated phenols, as is well known, react most strongly with the reagent when boiled. Use might be made of this differential character of the reaction to determine non-halogenated in the presence of halogenated phenols. Increase in the relative amount of reagent 1 employed considerably increases the amount of water-soluble coloured substance formed with the Millon reagent by non-halogenated phenol.

B. *Procedure with biological materials*

Proteins, fats, and some inorganic salts give a precipitate with Millon reagent and interfere with its use in colorimetry. However, quantitative separation of halogenated phenols from various interfering substances present in biological material can be effected by extraction with toluene, steam distillation, and precipitation with reagent 1. The use of the two latter procedures is illustrated below.

Determination of CX in urine. 2 ml. of urine are diluted with 4 ml. of water and then 2 ml. of reagent 1 are added. After 3 min. the mixture is centrifuged, 4 ml. of the clear supernatant fluid are transferred to a test-tube, and 0·3 ml. of reagent 2 added. The solution is allowed to stand for 30 min. at room temperature, cooled, extracted with 4 ml. of chilled ether, and colorimetry carried out as in the method for aqueous solutions. A standard curve is constructed from results obtained with water solutions of known *CX* content similarly treated. The sensitive range is that covered in the experiment described in Table 1.

Determination of CC in urine. 5 ml. of urine are made just acid to litmus, transferred to a long-necked distillation flask, and steam-distilled until 15 ml. of distillate have collected. Excessive foaming is prevented by the addition of a few drops of liquid paraffin. The distillate obtained is treated as in the procedure given for determination in water. A standard curve is prepared from results obtained with aqueous solutions of known content distilled in the same apparatus and treated similarly. The sensitive range is shown in Table 1.

Determination of CX in blood. 5 ml. of oxalated blood are deproteinized by mixing with 20 ml. acetone, filtered under suction, and the precipitate washed with 10 ml. acetone and 5 ml. ether. The pooled filtrates are quantitatively transferred to a long-necked distillation flask, joined to a water condenser and held over a water-bath. 0·3 ml. 40% NaOH and, to diminish foaming, a few drops of liquid paraffin are added. The acetone and ether are then driven off slowly by heating the water-bath. Evaporation is discontinued when, the constant flow of distillate having ceased, foaming becomes excessive. The residue of liquid is then rendered acid by addition of 1 ml. 25% (v/v) H_2SO_4, and steam-distilled. When 15 ml. of distillate have collected, the distillation is stopped. The distillate or a suitably diluted portion is used as in the method for

aqueous solutions. A standard curve is prepared by subjecting aqueous solutions of known concentration to similar treatment, using the same apparatus. The sensitive range is shown in Table 1.

The same procedure can be applied to the determination of *CC* in blood.

Determination of CX and CC in minced tissues. Extraction of *CX* and *CC* is carried out from minced tissue by boiling in acetone under a reflux condenser for 1 hr. Otherwise the procedure follows the method for blood.

distillation procedures is therefore a small and, within the error of estimation, a constant fraction of the total amount originally present. Hence the incomplete recovery on distillation does not invalidate the procedure. It is therefore important, however, to standardize the distillation procedure very accurately.

Specificity of estimation. The method is highly specific for halogenated phenols. The examination

Table 1. *Recovery of halogenated phenols from biological materials*

Test substance	Medium		mg./100 ml.						
p-Chloro-*m*-xylenol (*CX*)	Urine	Added	0	7	11	24	38	57	80
		Found	0	8	11	25	36	57	78
p-Chloro-*m*-xylenol (*CX*)	Blood	Added	0	2·0	4·0	4·0	6·0	6·0	—
		Found	0	2·0	3·8	4·0	5·6	5·7	—
p-Chloro-*m*-cresol (*CC*)	Urine	Added	0	5	10	15	20	25	—
		Found	0	5	11	15	20	25	—

Results. The analytical findings when halogenated phenols were added in known amount to urine and blood are seen from representative experiments summarized in Table 1. Within the range of the reported experiments the method can be relied upon to give results with a maximum error of about 5 %.

Table 2. *Influence of phenol on estimation of p-chloro-m-xylenol (CX)*

Composition of test mixture		Analytical finding	
CX (mg./100 ml.)	Phenol (mg./100 ml.)	*E*	Estimated *CX* (mg./100 ml.)
	(*a*) Solution in water		
1·6	0	0·40	1·6
1·6	4	0·40	1·6
1·6	7	0·41	1·6
1·6	14	0·45	1·8
1·6	28	0·61	2·4
3·2	0	0·84	3·2
3·2	16	0·82	3·1
3·2	32	0·86	3·3
3·2	49	1·14	4·3
0	28	0·10	0·4
0	56	0·20	0·8
0	160	0·70	2·7
	(*b*) Solution in urine		
20	0	0·35	20
20	20	0·35	20
20	40	0·36	21
20	100	0·39	22
20	200	0·41	23
20	400	0·53	30

The absolute recovery of chlorophenol in the methods involving separation by distillation was 93–98 % in these and similar experiments on *CX*, and 83–87 % for *CC*. The loss of chlorophenol in the

of 1000 urine samples from human subjects, none of whom had received chlorophenol prior to the test, failed to reveal a single urine showing a positive reaction for halogenated phenol. The blood of normal untreated humans is likewise consistently negative.

Tyrosine does not react with Millon reagent under the conditions of the test, nor do phenol and cresol in the concentrations present in urine. The degree of interference with the estimation of *CX* resulting from the presence of unhalogenated phenol is indicated in Table 2. The relative yields of the ether-soluble coloured substance, with *CX* and phenol respectively, are at least 50 : 1. Interference with *CX* estimation was marked only when the concentration of phenol was over 10 times that of *CX*. In the presence of *CX* there is a measurable increase in the intensity of the colour of the ether-soluble material formed with phenol. The effect is, however, small and is not a serious source of error. In the method used with urine, phenol interferes with the determination of 20 mg./100 ml. only at concentrations greater than 100 mg./100 ml. (Table 2 (*b*)). The normal physiological level of 'free phenol' in urine, however, is only 5–30 mg./100 ml.

SUMMARY

1. The application of the Millon reaction to the determination of halogenated phenols in the presence of phenol is described.

2. Semi-micro-procedures for the determination of *p*-chloro-*m*-xylenol and *p*-chloro-*m*-cresol in water, urine, blood, and minced tissue are given in detail.

We are indebted to the Teva Middle East Pharmaceutical and Chemical Works Co., Ltd., Jerusalem, for a supply of *p*-chloroxylenol.

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Fate of Halogenated Phenols in the Organism

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Halogenated phenols, long employed for external disinfection, are also powerful internal disinfectants [Zondek, 1942*a*]. As the result of parenteral or percutaneous application in man, halogenated phenols are excreted in the urine, and prevent its bacterial decomposition on storage. In the clinical treatment of pyelitis and cystitis, their application may effect sterilization of the urine [Zondek, 1942*b*]. Blood from treated patients is bacteriostatic and inhibits growth of inoculated staphylococci. Successful treatment of septic infections in man is thus made possible.

The present paper is concerned with the fate in the body of *p*-chloro-*m*-xylenol (4-chloro-3:5-dimethylphenol, referred to briefly as *CX*), *p*-chloro-*m*-cresol (4-chloro-3-methylphenol, referred to as *CC*), and with the following derivatives of *p*-chloroxylenol: acetate, benzoate, sulphate, methyl and ethyl ethers, and the *o*-iso-propyl derivative.

METHODS

The method of determining *CX* and *CC* in the presence of phenol, described by Zondek, Shapiro & Hestrin [1943], was used. *CX* and *CC* react with Millon reagent at room temperature, yielding coloured substances which can be extracted with ether and quantitatively determined therein. Phenol in the quantities present in body fluids gives no colour with Millon reagent under the conditions of these assays.

Glucuronic acid in urine was estimated according to Mozolowski [1940]. The quantity of sulphuric esters in urine was calculated by subtracting the free sulphate from the total sulphate (sulphate after acid hydrolysis).

RESULTS

Excretion of CX (p-chloro-m-xylenol) and CC (p-chloro-m-cresol) in the urine

In rabbits. Rabbits (approx. 1 kg.) were injected subcutaneously with 1 g. of *CX* dissolved in 10 ml. of olive oil. The urine was collected daily and its content of free *CX* determined. The pH was usually 7·5–8·2. About 15 % of the *CX* given was excreted during 4–5 days (Table 1). In an analogous experiment with *CC*, 15–20 % was recovered in the urine.

In man. *CX* administered intramuscularly to man is not usually excreted in the free form if the pH of the urine is acid. The difference between human beings and rabbits in respect of *CX* excretion was associated with the more acid reaction of human urine (pH 5·5–6·7). A *CX* content in urine as high as 50 mg./100 ml. and recoveries up to 8·3 % were obtained in man when the urine was made alkaline by administration of $NaHCO_3$. Concentrations of *CX* of 30 mg./100 ml. convert urine into a bactericidal fluid. If the urine is again rendered acid by administration of NH_4Cl, *CX* excretion ceases (see Table 2). But in spite of the fact that no *CX* could be demonstrated chemically, some bactericidal substance seems to have been excreted, since with most treated patients no ammoniacal

Table 1. *Excretion of p-chloro-m-xylenol in urine of rabbits*

Days after injection	Excretion of free substance by rabbit no. 1 (mg.)	2 (mg.)	3 (mg.)	4 (mg.)
1	43	40	10	30
2	46	60	50	28
3	50	30	44	32
4	25	10	34	30
5	12	—	6	8
6	—	—	—	—
Total excreted	176	140	144	128

Average excretion = 150 mg. = 15 % of quantity injected.

Table 2. *Effect of urinary* pH *on the excretion of p-chloro-m-xylenol in man*

Patient D.H.

Date	Material injected (g.)	Additional treatment	pH of urine	Material excreted (mg.)
17–18 Jan.	7	None	—	—
19–21	12	$NaHCO_3$	7·6	1320
22	0	,,	7·2	980
23	0	,,	7·1	300
24–26	0	NH_4Cl	5·2–5·8	60
27–28	8	,,	5·4	—
29	6	$NaHCO_3$	5·4	—
30	6	,,	7·2	240
31	0	,,	7·8	330
2 Feb.	3	,,	7·8	170
3	0	None	6·4	60
4	0	None	6·2	—
5	0	None	6·3	—
Total given	42	Total excreted = 3460 mg. = 8·3 %		

decomposition of the stored urine occurred. In other experiments decomposition of urine was found to be prevented by concentrations of *CX* exceeding 2 mg./100 ml.

When *CC* was administered to several human patients intramuscularly in oil, it was in no case recovered in the urine in appreciable quantity. It was extremely difficult to make the urine of these patients alkaline, an effect which was apparently due to the administration of *CC*. Where alkalinization was effected a negligible excretion of *CC* was observed.

Combined forms of CX in urine

Since phenol appears in urine combined with glucuronic acid and with sulphuric acid as well as in the free state, it was to be expected that *CX* too would be found partly combined in these forms. The combined *CX* was determined by estimating glucuronic acid and bound sulphuric acid before and after *CX* treatment. In humans injected with 5 g. *CX*, approximately 700 mg./100 ml. were excreted combined with glucuronic acid, and 840 mg./100 ml. as a sulphuric ester; 14 % of the quantity injected is thus found in the form of glucuronide, and 17 % as sulphuric ester. The total recovery of *CX* in combined form is thus 31 %.

Dissociation of combined CX by urine

Some urine samples, which give no chemical tests for free *CX* when fresh, may develop considerable quantities on standing. This finding was corroborated by bacteriological tests. Urine which contained *Escherichia coli* when fresh, became sterile after standing for some days, and in some cases then also destroyed added bacteria.

Urine specimens in which no free *CX* could be demonstrated were stored for 1 month. Control specimens were boiled before storing. Free *CX* was then determined, and 9–17 times as much was found in the unboiled urine as in the boiled specimens. These results suggest that the dissociating agent is destroyed by heat and is of an enzymic nature, presumably a glucuronidase or sulphatase. This point is under investigation.

Excretion of CX in urine after injection of CX esters and ethers

Ester derivatives of hormones have been of great value in hormone therapy (e.g. oestradiol benzoate, testosterone propionate, desoxycorticosterone acetate, etc.). The high activity of such esters is attributable to their slow absorption on subcutaneous administration and the consequent sustained liberation of active substance in the body. It seemed possible that in phenol-chemotherapy, too, the use of ester derivatives would be advantageous. The acetic acid and benzoic acid esters of *CX* were less toxic in animals and more soluble in oil than was *CX* itself (solubilities—20 and 25 % respectively for the esters, as against 10 % for *CX*). These ester derivatives could therefore be safely administered in larger single doses. It was found, also, that *CX* dissolves in oil to a higher concentration in the presence of *CX*-esters. In oil containing a total of 30 % of *CX*-acetate, for instance, 20 % of *CX* could be dissolved as against only 10 % in pure oil. The sulphuric acid ester presented a particular advantage, its sodium salt being soluble in water up to 5 %. *CX*-methyl- and ethyl-ethers were liquids at 30° and could be injected without dilution.

It was thus of interest to ascertain whether esters and ethers of *CX* are decomposed in the body with regeneration of active *CX*. The excretion of free *CX* in rabbit urine after injections of the ester serves as an indication of such regeneration. The *CX*-esters and ethers do not give a positive colour reaction with Millon reagent.

CX-acetate and benzoate are readily absorbed and decomposed to *CX* in the animal body (Fig. 1).

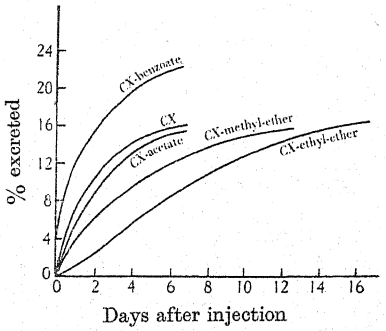

Fig. 1. Total amount of *p*-chloro-*m*-xylenol (*CX*) excretion in rabbits, as % of amount injected.

The total amount of free *CX* excreted in urine in such cases is quite as high as when *CX* itself is injected, though the rate of excretion is somewhat slower.

CX-methyl- and ethyl-ethers are also decomposed to give free *CX*, the extent of the excretion of *CX* being equal to that obtained when *CX* itself was injected. This is of particular interest, since it is not generally accepted that the animal organism can perform such dealkylations. Excretion of free *CX*, after the ethers had been given, continued for 11–17 days, as against a period of excretion of 4–6 days when free *CX* or *CC*, and esters of *CX*, were tested. Repetition of the experiment with various modifications consistently confirmed this finding. The slow absorption or decomposition of the test substance should be of clinical importance.

After injection of *CX*-sulphate no free *CX*, or only small amounts, appeared in the urine. *CX*-sulphate, which is a detoxication product of *CX*, is

presumably rapidly excreted, largely in an unchanged condition. After *o*-iso-propyl-*CX* had been given to human beings or to rabbits it was not found in the urine. Its water solubility is low and it is also practically insoluble in dilute alkali.

Concentration of CX in blood

Concentrations in blood of 3–10 mg./100 ml. are observed only when large amounts of *CX* have been given, e.g. 50 g. to man or 1–3 g. to a rabbit. These large doses can be administered by inunction of a 30 % *CX*-ointment. Concentrations in blood of 1 mg./100 ml. were obtained when 1 g. of *CX* was given to man by intramuscular injection. The maximal *CX* content was observed 1–5 hr. after treatment, but in most cases no *CX* was chemically demonstrable in the blood. Nevertheless, we have sufficient evidence from the urinary data of the completeness of the absorption of *CX*. Evidence was also provided by the *CX* content of cantharidin blisters in *CX*-treated patients. Despite our inability to detect *CX* in the blood by chemical means, we have found that the blood of the treated patients showed considerable bacteriostatic power towards inoculated cocci. Whereas chemically detectable quantities of *CX* disappear from the blood several hours after treatment, bacteriostasis sets in only after more than 24 hr. The cause of this disparity will be dealt with elsewhere.

CX in cerebrospinal fluid

In no instance were chemically detectable quantities of *CX* present in cerebrospinal fluid taken from rabbits or from men at various intervals 4–48 hr. after *CX* treatment.

Fate of CX injected in the body

As excretion in the urine of free and combined *CX* accounted for only part of the *CX* injected, it was of importance to establish what proportions of the amount administered are excreted in the faeces and retained in the body. Five rats (70–80 g.) were given 100–200 mg. of *CX* by injection, and the urine and faeces collected for 48 hr. The animals were then killed, minced and extracted first with cold and then with warm acetone, and *CX* determined in the extracts. About 15 % of the *CX* injected was recovered in the faeces. Half an hour after injection, 85–90 % of the *CX* was still present in an unchanged form in the whole body. After 48 hr. only 10 % could be recovered from the minced carcass. The total quantities of *CX* found in urine (combined + free = approx. 30 %), faeces (15 %), and in the body (10 %) after 48 hr. thus accounted for only approx. 55 % of the quantity injected. It must be assumed that some part of *CX* injected is changed by the body to a substance which cannot be detected by our procedure for the estimation of *CX* in tissues.

Absorption of injected CX and its distribution in the body

200 mg. of *CX* in 2 ml. olive oil were injected subcutaneously into the left forelegs of five rats weighing 100–120 g. After 24–48 hr. the animals were killed. The posterior and anterior parts of each were minced and analyzed separately for *CX*. The bulk of the recovered *CX* was found in all the animals in the anterior part of the body where the injection had been given. In one typical experiment, 24 hr. after the administration, 60 % of the injected quantity was found in the anterior part and only 5 % in the posterior. After 48 hr. the anterior part contained 6 % and the posterior 1 % of the quantity injected. Absorption of *CX* is therefore a comparatively slow process.

SUMMARY

1. When *p*-chloro-*m*-xylenol (*CX*) is injected into rabbits, approximately 15 % of the amount administered is excreted in urine. In human urine of acid reaction only very small quantities (about 2 mg./100 ml.) are excreted. These quantities are usually sufficient to inhibit ammoniacal fermentation of the urine. When human urine is rendered alkaline by administration of $NaHCO_3$, *CX* concentrations up to 50 mg./100 ml. are obtained, and up to 8 % of the administered *CX* is excreted. Concentrations of *CX* above 30 mg./100 ml. render the urine bactericidal.

2. After administration to human beings or to rabbits *o*-iso-propyl-*CX* is not excreted in the urine.

3. *p*-Chloro-*m*-cresol injected into rabbits is rapidly excreted in the urine. In man no excretion was observed.

4. The acetic acid and benzoic acid esters and the methyl- and ethyl-ethers of *CX* decompose in the body with regeneration of *CX*, which is excreted in the free form in the urine. The absorption of the esters is somewhat slower than that of *CX* itself. The time required for complete absorption and excretion of *CX*-ethers is three times that for *CX* itself.

5. *CX* is partly (30 %) excreted in the urine as glucuronide and sulphate ester (in equal amounts) after administration to man.

6. Even when large doses are administered the concentration of *CX* in blood seldom exceeds 1 mg./100 ml. Concentrations of 3–10 mg. are encountered occasionally, but only when very large amounts are given. The maximum quantities in blood are found 1–5 hr. after treatment.

7. No *CX* was detected in cerebrospinal fluid after administration of this substance.

8. The aggregate amount of *CX* found in the urine and faeces and recovered from the animal body after 48 hr. accounted for only approximately 55 % of the amount of *CX* given.

9. The absorption of injected *CX* is a slow process and is incomplete even 48 hr. after the injection.

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The Replenishment of Depleted Skeletal Reserves of Magnesium

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It has been shown that the Mg of the skeleton is mobilized in response to the threatened shortage of the element in the soft tissues that results from dietary insufficiency of Mg [Duckworth, Godden & Warnock, 1940]. In the face of the persistent demand for Mg in Mg deficiency it was found that the rate of skeletal growth determined the amount that could be liberated on account of the demand of the skeleton itself for Mg [Duckworth & Godden, 1941]. The most striking feature of the mobilization of Mg was the high speed with which large quantities were released.

The question that immediately suggests itself is whether the skeletal stores can be replenished as easily as they are depleted. The problem is of practical interest in the nutrition of farm animals and man, in which the dietary intake of Mg is liable to vary. This is especially true in the case of man for whom vegetables, the supply of which fluctuates throughout the year, are the most important single source of Mg [Duckworth & Warnock, 1942–3]. The result is that varying degrees of inadequacy or alternating periods of adequacy and inadequacy are more common than constant minor degrees of insufficiency. This state may be further accentuated by losses in sweat, which may become abnormal owing to seasonal change in temperature or conditions and intensity of work [Duckworth & Warnock, 1942–3].

The present study was undertaken to examine this problem, and because of the profound influence of the rate of bone growth on liberation of skeletal Mg, a combination of Mg deficiency with different degrees of Ca deficiency was used. This approach also allowed the examination of certain points of interest concerning the interrelation of Ca and Mg in the skeleton.

METHODS

Diets used. The preparation of the diet, the methods of analysis and the management of the animals have been reported [Duckworth *et al.* 1940; Duckworth & Godden, 1941] and need not be repeated here. The technique for preparing a diet low in Mg simultaneously removes the Ca with about the same degree of efficiency, so that no additional procedure is required. The diets contained less than 6 parts per million (p.p.m.) of Mg and about 1–2 p.p.m. of Ca.

Four diets were prepared, each with a P content of 0·35 %. By additions of $CaCO_3$, with slight compensatory adjustments in the starch content, these diets were arranged to contain 0·90, 0·30, 0·10 and 0·00 % of Ca, and are referred to as diets A, B, C and D respectively.

Animal management. One litter of eight rats (4 ♂ and 4 ♀) at weaning was allotted to each diet. Of each litter six rats were placed on the appropriate deficient diet while two remaining rats of the litter were given the same diet supplemented with sufficient $MgSO_4$ to raise the Mg content to 0·07 %. The six animals undergoing skeletal Mg depletion were observed closely, and upon the death of one member of the set another, judged clinically to be close to death, was dosed with several drops of a solution of $MgSO_4$ and then transferred to the same diet supplemented with Mg. Thus of each litter three animals (hereafter referred to as 'depletion rats') died of Mg deficiency and three others (subsequently referred to as 'curative rats') were sacrificed after each had received a curative period of 10 days on the appropriate Mg-supplemented diet. The two remaining animals were treated as controls, one being killed at what was estimated to be the average

time of survival of the three depletion rats and the other being killed 10 days later. These animals are referred to as the 'depletion control' and the 'curative control' respectively.

The animals were under constant supervision during the day and were visited every 4 hr. during the night. Thus differences between the corresponding depletion and curative animals, from the standpoint of length of time on the Mg deficient diets, were minimized.

RESULTS

The average values for bone composition are given in Table 1. Individual values for the percentage of Mg in bone ash are given in Fig. 1.

Table 1. *Composition of bones of rats receiving different types of diets*

Group*	Wt. of bones (mg.)	Wt. of ash in bones (mg.)	Wt. of ash in bones (%)	Wt. of Mg in ash (mg.)	Wt. of Mg in ash (%)	Mg deposited during curative period (mg.)
A —	362	204	56·4	1·14	0·56	
A —+	524	312	59·5	2·46	0·79	1·32
A 1 +	350	201	57·4	1·94	0·97	
A 2 +	544	332	61·0	3·00	0·90	
B —	363	195	53·7	1·10	0·57	
B —+	565	327	57·9	2·51	0·77	1·41
B 1 +	370	207	55·9	2·00	0·97	
B 2 +	642	374	58·3	3·19	0·85	
C —	388	191	49·2	1·06	0·56	
C —+	461	235	51·0	1·95	0·83	0·89
C 1 +	406	208	51·2	2·08	1·00	
C 2 +	448	227	50·7	2·12	0·93	
D —	279	135	48·4	0·76	0·56	
D —+	266	116	43·6	1·08	0·93	0·32
D 1 +	268	122	45·5	1·31	1·07	
D 2 +	320	130	40·6	1·42	1·10	

* — indicates depletion rats; —+ indicates curative rats; 1 + indicates depletion control rats; 2 + indicates curative control rats. Letters indicate the diet used (see 'Methods').

The average survival periods of the depletion rats on Mg-deficient diets A, B, C and D were, respectively, 5·0, 7·3, 8·7 and 11·3 days. Comparison of the data for bone growth should be made with these differences in mind. Since a more extensive study has already been published [Duckworth & Godden, 1941] of bone development in rats receiving the present diets, further discussion is unnecessary.

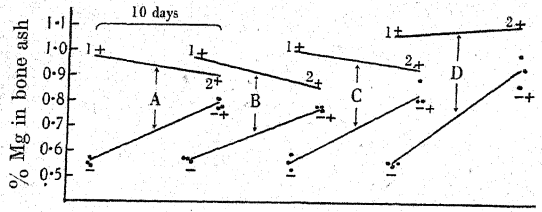

Fig. 1. Percentages of Mg in the bone ash of rats receiving different diets and treatment. — indicates depletion rats; —+ indicates curative rats; 1+ indicates depletion control rats; 2+ indicates curative control rats. Letters indicate the diet used (see 'Methods').

The percentage of Mg in the ash was about the same in all depletion rats irrespective of diet. After 10 days of re-alimentation with Mg the influence of different degrees of Ca adequacy became apparent. Thus the curative rats receiving diets A and B showed closely similar percentages of Mg in the ash, but the curative rats receiving diets C and D showed progressively higher concentrations with decreasing Ca intake. It will be noted that the individual values were not so closely spaced in the two latter groups. The control rats receiving diets A, B and C showed the characteristic reduction of Mg percentage with increasing age. The control rats receiving diet D did not show this reduction and the values were above those of controls receiving the normal diets A and B. There is, perhaps, some indication of increased Mg percentage in the control rats receiving diet C. In none of the curative animals was the value of the corresponding curative control reached, although one rat on diet C and all on diet D approached, or surpassed, the curative control values for diets A and B.

The amounts of Mg in the bone were closely similar for corresponding rats of diets A and B. The curative rats and the curative control of diet C showed, by the lower quantities of Mg and ash, the influence of restricted Ca intake that had not been indicated in the depletion rats and the depletion control, which had received the diet for a shorter period. The amounts of Mg in the bones of rats receiving diet D were lower in all cases than for corresponding rats on other diets.

The quantities of Mg stored by the curative rats, estimated by difference between the values for depletion and curative animals, were about the same in rats of diets A and B. The value was less in animals receiving diet C and least in those receiving diet D.

The symptomatology, including cessation in growth, did not differ from that of previous studies. After the addition of Mg, curative rats of diets A and B grew normally, rats of diet C showed subnormal growth and those of diet D slight increases.

DISCUSSION

The regularity of the individual values for the percentage of Mg in bone ash, given in Fig. 1, indicates that the differences observed in the curative littermates at the time they were killed can be attributed to differences in the degree to which Mg is stored under different dietary conditions. This is supported

by the close similarity in the results observed for the curative rats receiving diets A and B, which are essentially normal diets.

It is well known, and the evidence has been summarized elsewhere [Duckworth, 1938–9], that when demineralization of the skeleton occurs the Mg content of the bone ash generally increases. Because of the proportional increase in organic matter, itself containing Mg, during demineralization, it has not been possible to state categorically that the increase found in the percentage of Mg in the bone ash arose from an increase in the Mg content of the crystals of bone salt. The possibility that the increase was the outcome of a greater amount of organic matrix remained.

The present findings contribute information about this aspect of the interrelation of Ca and Mg in bone. The Mg content of the ash of the control animals increased as the Ca supply diminished, the customary reduction with age giving way to an increase with progressive demineralization in the case of diet D. In the depletion rats this did not happen, irrespective of the degree of calcification of the bones. The curative rats receiving diets C and D showed progressively greater percentages of Mg in the ash as Ca intake fell. The simplest explanation of these changes is that Mg can replace Ca, to a limited extent, in the bone salt crystal during periods of Ca insufficiency. If, however, there is simultaneously a deficiency of both Ca and Mg, then this replacement does not occur. When, after a period of both Ca and Mg lack, the Mg is restored to the diet, it then begins to exert its Ca-sparing effect in the skeleton. It cannot be argued convincingly that the increase in the percentage of Mg in the ash of rats receiving diet D arose in any way from the greater percentage of organic matter. This would imply that the organic matrix of bone contained many times more Mg/unit weight than other tissues [the data are collected in the review of Schmidt & Greenberg, 1935] and that this higher content could be dispensed with in Mg deficiency to give the constant results found for the depletion rats.

Tufts & Greenberg [1937–8*a*] showed that the Mg content of soft tissues is not reduced in Mg deficiency. Thus, when Mg is restored to the diet after a period of deficiency, there is no soft tissue deficit to remedy, and the only requirements are those for the replenishment of the skeletal reserves and for new soft tissue and skeletal growth. Since Tufts & Greenberg [1937–8*b*] obtained good growth, normal gestation and parturition, and fair lactation, in rats whose diet contained only 0·005 % Mg, it is unlikely that the present diet failed to supply sufficient Mg to meet the above demands. This failure of the curative rats to attain the same percentage of Mg in the ash as the corresponding curative control rats may have arisen from either of two main causes, or from a combination of them. The first cause of failure may be that Mg cannot re-enter the depleted crystal, or that it can only re-enter it at a very slow rate. The second may be that new bone salt deposited when Mg is restored to the diet does not initially contain the normal quantity of Mg, normal composition being attained only gradually. That this failure almost certainly involves the former of these alternatives is shown by earlier studies [Duckworth & Godden, 1941], where it was found that the return of plasma Mg to normal levels during the second phase of the deficiency, when drafts on the skeletal Mg deposits have ceased, was not accompanied by a return of Mg to the skeleton. A further opportunity for Mg to enter bone salt would be during those processes of solution and redeposition of bone salt which, although detected, have yet to be measured accurately. This additional process was not considered above when the replenishment of the reserves of the growing animal was discussed, but it may have been mainly responsible for the re-entry of Mg into the bone salt, in the curative rats receiving diet D in which no bone growth occurred.

Irrespective of the manner in which Mg returns to the depleted bone salt it is clear that the reparation of the deficit is less rapid than the depletion process. Attention has been called elsewhere [Duckworth & Warnock, 1942–3] to the wide variations in the concentrations of Mg in human bone, and to the evidence of dietary deficiencies in some social groups presented. It may be that the variation noted is evidence not only of depletion but also of the slow rate at which the deficit is made good when Mg supplies become adequate. It might be expected that in the adult the return of bone composition to normal, in respect of Mg, would require longer than in the young growing animal, because of the absence of active bone growth. In this case, however, the mechanism involved may resemble that of the curative rats of diet D, and may depend on the processes of solution and re-precipitation mentioned above.

SUMMARY

1. Evidence is presented to show that the increase of Mg in bone ash which occurs in demineralization arises from a Ca-replacing action of Mg. It does not occur if Mg supplies are inadequate.

2. The replenishment of skeletal Mg reserves, in rats depleted by acute Mg deficiency, is incomplete after a 10-day period of re-alimentation with Mg, irrespective of whether or not bone growth occurs.

3. The interrelation of Ca and Mg in the skeleton is discussed in the light of the experimental findings

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The Iodometric Micro-determination of Arsenic in Biological Material

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The method of Allcroft & Green [1935; see also Kiese, 1937] for the titrimetric estimation of arsenic in tissue digests yielded satisfactory results with 0·05–1 mg. As. As is separated as AsH_3, which is passed into $AgNO_3$ solution, the arsenite formed being titrated with dilute I_2. It has been found possible to adapt this technique for 5–50 μg. As by reducing the volume of the solution to be titrated till the I_2 required for a distinct colour change is a small fraction of that required to oxidize the arsenite.

Tubes have been constructed in which 1·5 ml. N/50 $AgNO_3$ are sufficient for efficient absorption of AsH_3 at the rapid rate of passage of H_2 necessary for complete separation of As as AsH_3. Titration with N/200 I_2 is carried out in the absorption tube. Under the conditions of the experiment, a distinct colour change is produced by 0·003 ml. I_2, although the amount may vary slightly with the observer and with the lighting. The microburette described by Conway [1939], with a ground-glass joint in place of the rubber stopper in the standard model, is used in the titration. If the reservoir is shielded from light, there is little change in the I_2 over intervals of weeks.

The procedure described has been found reliable in thousands of routine estimations carried out by several workers in these departments and elsewhere. No difficulty has been encountered in adapting it to many types of material.

OUTLINE OF METHOD

Organic matter is destroyed by digestion with a mixture of HNO_3, $HClO_4$ and H_2SO_4. The digest is treated with $(NH_4)_2C_2O_4$ to remove interfering N compounds. When the biological material is rich in As and the sample taken for analysis is small ('Digestion—small scale'), AsH_3 is evolved directly from the digest. With large samples of material ('Digestion—large scale'), As is distilled from the digest as $AsCl_3$, and AsH_3 is evolved from the acid distillate. The procedure thenceforth is the same in both cases. After adjusting the acid concentration and adding $CuSO_4$ and $SnCl_2$ to catalyze H_2 evolution, Zn is introduced into the As-containing solution and the gas liberated is passed through 10N NaOH and N/50 $AgNO_3$. The dimensions of the $AgNO_3$ tube are shown in Fig. 1. To avoid risk of breaking the tip of the Conway burette, the gas inlet (thick-walled capillary tubing of 1 mm. internal diam.)

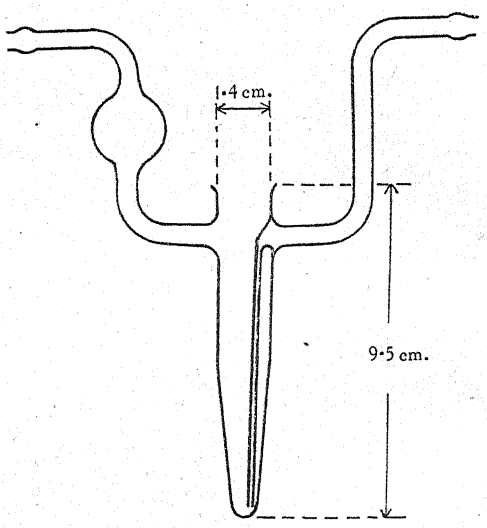

Fig. 1. The absorption tube.

should be situated close to the wall, as shown. Before titrating the arsenite in the absorption tube, solid KI is added to remove excess Ag^+ which would otherwise prevent the appearance of the I_2-starch colour. This is followed by $NaHCO_3$ to remove HI produced in the titration, and starch indicator. The tip of the Conway burette is immersed in the solution, which is stirred during the titration by passing air through the gas inlet of the absorption tube.

Since a large number of digestions can be carried out simultaneously, the time involved per sample at this stage of the determination is negligible. By working in serial fashion with six sets of apparatus, estimations can be put through the stage of AsH_3 separation and arsenite titration at about 10 to the hour. When preliminary separation of As as $AsCl_3$ is necessary, the distillations can be done at the rate of one every 10 min. by using duplicate apparatus.

EXPERIMENTAL

Reagents

All reagents should be of analytical or As-test quality

HNO_3, conc.	$SnCl_2$, 40% in conc. HCl
$HClO_4$, 60% (w/w)	$CuSO_4$, $5H_2O$, 5%
H_2SO_4, conc.	Zn shot, freed from dust and extra large shot
HNO_3, fuming	H_2SO_4, 20% (w/v)
$(NH_4)_2C_2O_4$, sat. solution	KI, cryst.
$FeSO_4$, $7H_2O$, cryst.	$NaHCO_3$, powdered
KBr, cryst.	Starch solution, fresh
NaCl, cryst.	I_2, N/200
$AgNO_3$, N/50	Standard arsenite solution, prepared from As_2O_3
NaOH, 10N	
Glycerine	

Procedure

Digestion—small scale. Up to 5 ml. blood, 10 ml. urine or 2 g. tissue are measured into a 100 ml. Kjeldahl flask, and conc. HNO_3 (5 ml.), $HClO_4$ (3 ml.), conc. H_2SO_4 (10 ml.) and two or three clean glass beads are added. The flask is warmed, with shaking, over a naked flame till brown fumes are given off, and heating is continued on a digestion stand over a very small flame (to avoid bumping) till they are no longer evolved. The contents are then gently boiled till H_2SO_4 fumes appear, and the clear liquid becomes colourless. Heating is continued for 10 min. If the mixture chars at any time, the flask is cooled slightly and a few drops of fuming HNO_3 added.

When digestion is complete, the flask is allowed to cool and $(NH_4)_2C_2O_4$ solution (5 ml.) is added. The liquid is brought to the boil and heating continued till white fumes appear. Distilled water (5 ml.) is added to the cooled liquid, which is again brought to the boil, and heating continued till 5 min. after the first appearance of white fumes.

Control experiments, with reagents alone, are carried out in exactly the same manner. It is important that heating of the digest at each stage should be continued for at least the period specified.

Digestion—large scale. Tissue. Up to 20 g. of minced, moist tissue are wrapped in very thin paper and placed in a 300 ml. Kjeldahl flask (with ground joint). After the addition of conc. HNO_3 (30 ml.) and two or three clean glass beads, the flask is set aside overnight.

Blood. Up to 30 ml. blood are treated as described above.

Urine. To not more than 150 ml. urine one-fifth its volume of fuming HNO_3 and two or three glass beads are added, the liquid evaporated to about 10 ml., and the digestion then carried out.

$HClO_4$ (15 ml.) and conc. H_2SO_4 (20 ml.) are added to the flask, which is warmed cautiously with shaking over a naked flame till reaction sets in, and is then placed on a digestion stand. After the first violent reaction subsides, the burner is lit and the contents brought to the boil. When digestion is complete, 20 ml. of $(NH_4)_2C_2O_4$ solution and of water are added. It may be necessary to make several additions of fuming HNO_3 before digestion is complete.

$AsCl_3$ *distillation.* This is necessary only after large-scale digestion. A delivery tube is connected, with conc. H_2SO_4 as lubricant for the ground-glass joint, to a 150 ml. Fresenius flask containing distilled water (40 ml.) immersed in ice-water [see Bang, 1925].

Distilled water (50 ml.) is added to the Kjeldahl flask and the contents are cooled thoroughly. Through a wide-bore funnel, $FeSO_4$, $7H_2O$ (8 g.), KBr (2 g.) and NaCl (25 g.) are added and the flask immediately connected to the previously lubricated joint of the delivery tube. A 200 ml. volumetric flask is placed over the exit of the Fresenius flask. The liquid in the Kjeldahl flask is boiled vigorously for exactly 10 min., the ice around the receiver being renewed as necessary. With the liquid still boiling, the delivery tube is disconnected from the Kjeldahl flask. The contents of the Fresenius flask are then transferred to the 200 ml. volumetric flask, and, when cool, diluted to the mark.

Separation of As *as* AsH_3. Two absorption tubes (Fig. 1), the second of which acts as guard tube, are charged with N/50 $AgNO_3$ (1·5 ml.). The tubes are stoppered and connected to a small wash-tube containing 10N NaOH (renewed after 12 estimations), glycerine being used as a lubricant for rubber tubing. A right-angle tube, bearing a stopper to fit the AsH_3 generation flask, is attached to the wash-tube.

After a small-scale digestion, the contents of the Kjeldahl flask are transferred to a 150 ml. conical flask, with 40 ml. distilled water as washing fluid. Alternatively, a measured fraction of the diluted digest is made up to 50 ml. with 20% H_2SO_4. 40% $SnCl_2$ (10 drops) and 5% $CuSO_4$ (3 drops) are added. The liquid is warmed if necessary, Zn shot (15 g.) quickly introduced, and the flask at once connected to the previously prepared absorption apparatus.

After a large-scale digestion, if the portion taken is less than 100 ml., it is made up to this volume in a 150 ml. conical flask with 20% H_2SO_4. When the total distillate is used, it is transferred to a 250 ml. conical flask. 40% $SnCl_2$ (10 drops/100 ml. total vol.) and 5% $CuSO_4$ (3 drops/100 ml. total vol.) are added and the liquid is warmed. Zn (25 g.) is introduced and the flask immediately connected to the absorption apparatus.

Titration of arsenite. When H_2 has been passed through the absorption apparatus for 30 min. the tubes are disconnected. The rubber stopper of the absorption tube is washed down with a few drops of distilled water, and the gas inlet is connected to the compressed air. KI is added till the precipitated AgI redissolves. After the addition of a spatula-point of $NaHCO_3$ and a drop of starch solution the tube is slipped over the jet of the Conway burette. It is held in place by sliding the titration platform under it, and with air passing through the liquid at a gentle rate I_2 is run in till a red-brown colour appears and persists for 30 sec. Before the end of the titration, the gas exit is washed down by increasing the flow of air till a little of the liquid is blown into it. A daylight lamp is of advantage in observing the end-point. If the liquid in the guard tube darkens, it is titrated in the same way.

At the beginning of each day, the glass tubing in the burette is thoroughly washed out with fresh I_2 from the reservoir. To standardize the I_2, two different quantities of arsenite solution are measured into absorption tubes, the volume made up to about 2 ml. with N/50 $AgNO_3$, and the titrations carried out as described above. The amount of As (μg.) is plotted against ml. I_2, and the analytical results are read off from the straight line joining the two points. This line cuts the I_2 axis at the volume required to cause a colour change in 2 ml. N/50 $AgNO_3$ treated as above, in absence of arsenite.

RESULTS

In 20 experiments in which 20 μg. As were added as Na_2HAsO_3 to 100 ml. 20 % H_2SO_4 and separated as AsH_3, the mean recovery was 19·6 μg., and the standard deviation of a single observation from the mean was 0·73 μg. In the subsequent pages of this paper, 'standard deviation' always means the standard deviation of a single observation from the mean. In 26 determinations of 20 μg. As added to 3 ml. blood for small-scale digestion, average recovery was 18·9 μg. (standard deviation 2·3 μg.). These two series of estimations were carried out by a technical assistant who understood that he was dealing with amounts of As differing slightly from each other.

In 19 analyses in which three portions of the same digest, averaging 12·1 μg. As, were taken for the final stage (separation of AsH_3 and titration of arsenite) of the estimations, the standard deviation was 0·50 μg. As. A research worker investigating the procedure found the average recovery in 24 determinations of 20 μg. As added to 1 ml. blood to be 18·8 μg. (standard deviation 2·5 μg.). The results in Table 1 for As added to 20 g. of minced mouse illustrate the recovery after large-scale digestion and preliminary separation of As as $AsCl_3$.

Table 1. *Recovery of arsenic added to minced mouse prior to large-scale digestion*

As added (μg.)	As recovered (μg.)	Recovery (%)	As added (μg.)	As recovered (μg.)	Recovery (%)
8·0	7·4	93	48·0	46·3	97
10·0	9·3	93	50·0	45·8	92
12·0	12·4	103	56·0	55·6	99
16·0	17·8	111	60·0	54·7	91
20·0	17·5	88	64·0	60·1	94
30·0	32·2	107	72·0	63·4	88
32·0	30·6	96	72·0	69·4	96
40·0	42·4	106	80·0	77·2	97
40·0	38·1	95	80·0	77·1	96
40·0	34·4	86	100·0	96·6	97
48·0	47·6	99			

The accuracy is largely determined by the care taken in carrying out the control experiments with reagents alone. The lower limit for accurate estimations depends upon how much As is present in the reagents, the lighting during titrations and the sensitivity of the individual to the end-point. Below 10 μg. As, the titration error (about 0·3 μg.) assumes increasing importance as the amount of As decreases. The percentage As not retained in the first $AgNO_3$ tube varies with the rate at which H_2 is developed, but as a rule little AsH_3 reaches the guard tube with less than 50 μg. As or passes through it with less than 100 μg. present. With special care it is possible to work in the range of 2–10 μg. As with the accuracy found for larger amounts of the element.

DISCUSSION

Allcroft & Green [1935] found HNO_3 and $HClO_4$ to be the most satisfactory reagents for digestion in their As determinations. Their figures, however, showed a constant loss of As, particularly marked with 5 and 10 μg. As_2O_3, which should probably be attributed to the digestion rather than to titration or separation of AsH_3. In trials carried out here, poor recoveries were invariably obtained when H_2SO_4 was omitted from the digestion mixture. With a mixture of all three acids [Kahane, 1932; Hinsberg & Kiese, 1937; Cassil, 1937], loss of As during the combustion has not been noted, even though no special precautions were taken when fairly large amounts of Cl^- were present [see How, 1938; Klein, 1920].

The Sub-Committee of the Society of Public Analysts on the Determination of As [1930], Winterfeld, Doerle & Rauch [1935] and Kiese [1937], all found it necessary to carry out a double separation of As after digestion of large samples of biological material. NaCl is employed as a source of gaseous HCl in the preliminary separation of As as $AsCl_3$. Bang [1925] recommended KCl. T. B. B. Crawford & I. D. E. Storey (private communication) found a 20 % loss of As when HCl [Cox, 1925; Ramberg, 1921; Winterfeld *et al.* 1935; Kiese, 1937] was used. With small amounts of blood or urine, Dr Ena Brown (Mrs G. M. Henderson) has dispensed with the $AsCl_3$ distillation without increasing the error. The separation of As as AsH_3 directly from acid digests was recommended by Allcroft & Green [1935].

The most satisfactory reaction with Zn, in presence of Cu^{++} and Sn^{++}, for the separation of As as AsH_3, is obtained with 4N H_2SO_4. A similar rapid, but not violent, evolution of H_2 from the $AsCl_3$ distillate is obtained after it has been diluted to 200 ml., giving a concentration of HCl of approximately 2N. The use of NaOH to remove H_2S from the stream of AsH_3 and H_2 was recommended by Hinsberg & Kiese [1937]. According to Kiese [1937], N/50 $AgNO_3$ is sufficiently dilute to avoid the formation of any arsenate in the reaction with AsH_3. Our results indicate that both the separation of As as AsH_3 and the conversion into arsenite proceed quantitatively. Contrary to the suggestion of Conway [1939], we have found air stirring to be entirely suitable for iodometric titrations, in spite of the volatility of I_2.

Methods based on the matching of stains have been considered unsatisfactory by many authors, e.g. Winterfeld *et al.* [1935], Cassil [1939]. Thus Barnes & Murray [1930] found a standard deviation of 4·8 μg. in 150 estimations of 27·3 μg. As_2O_3, and Neller [1929] a 15 % standard deviation in the recovery of As (22–94 μg. As_2O_3) by a similar method.

In these studies, the length of stain produced by AsH_3 on $HgBr_2$ paper was measured and the corresponding figure for As read from a graph obtained with standard solutions. It should be noted that variation in length of stain for a given amount of As enters into the preparation of the graph as well as into analysis of an unknown sample, thus introducing the possibility of further error in the final result. The standard deviation of a single determination of 20 μg. As by the micro-titrimetric procedure was 3·7 %. While it is as sensitive as the usual Gutzeit procedure, the titrimetric technique is much more objective and does not call for the same careful control of physical conditions in the evolution of AsH_3.

Cassil & Wichmann [1939] have reported a procedure for small quantities of As very similar in principle to that described above. As was separated as AsH_3 and passed into 1 ml. 1·6 % $HgCl_2$. I_2 was added and the excess back-titrated with arsenite, in contrast to our direct titration. The final volume of solution titrated was about 8 ml. After the absorption of AsH_3 in $HgCl_2$, one atom of arsenic was found to correspond to eight atoms of iodine. The sensitivity of the estimation must, therefore, have been identical with that observed in titrating, in a volume of 2 ml., the product of the reaction between AsH_3 and $AgNO_3$, which requires two atoms of iodine for each atom of arsenic. It seems that the sensitivity in using $HgCl_2$ could be further increased by decreasing the volumes of the reagents added before the back-titration. Cassil & Wichmann's accuracy was somewhat greater than that claimed for the present procedure.

SUMMARY

1. With a special tube for the absorption of AsH_3 in $AgNO_3$ solution and the Conway micro-burette for the titration of the arsenite formed, the iodometric estimation of As in digests of biological material was modified to cover the range 5–50 μg.

2. The recovery of 20 μg. As was 98 %, the standard deviation of a single observation from the mean being 3·7 %.

3. Methods for the digestion of As-containing material were examined, and the use of a mixture of HNO_3, $HClO_4$ and H_2SO_4 adopted.

4. Preliminary separation of As as $AsCl_3$ from the digest was found to be necessary only when large samples of material were taken for analysis.

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The Components of the Antigenic Complex of *Salmonella Typhimurium*

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Recent work on the antigenic complex of *Eberthella typhosa* (*Bact. typhosum*) has shown that the substance may be obtained in a state approaching chemical homogeneity by trichloroacetic acid or diethylene glycol extraction of the bacterial cells, and by tryptic digestion of the dried organisms. The complete antigenic complex may be broken down by mild acetic acid hydrolysis with the libera-

tion of the component polysaccharide, conjugated protein and lipid constituents. The polysaccharide, which is non-antigenic and relatively non-toxic, is responsible for the specific properties of the intact antigenic complex. It has been shown to be free from lactose, pentose and uronic acid residues, but it yields 40 % *d*-glucose, 21 % *d*-mannose and 17 % *d*-galactose on hydrolysis. The polysaccharide has a minimum molecular weight of the order of 10,000 [Freeman & Anderson, 1941; Freeman, 1942]. Henderson & Morgan [1938] have shown that the antigenic complex of the O 901 strain of *Eberth. typhosa* is obtained by diethyleneglycol extraction of the organisms, and exists as a polymolecular complex containing three main components, a specific polysaccharide, a protein and a phospholipin.

In the present paper the methods which have proved successful in the case of *Eberth. typhosa* have been applied to the investigation of the nature of the components of the antigenic complex of *Salmonella typhimurium* (*Bact. typhi-murium*).

The results have in general confirmed those already obtained with the closely related *Eberth. typhosa*, but quantitative differences, especially in the nature of the specific polysaccharides, which account for the immunological dissimilarity of the two species, have been found.

EXPERIMENTAL

The organism was an O-strain (L.S.H.T.M. Catalogue C 3/23), which was free from Vi antigen. It was grown on a synthetic medium as described by Freeman, Challinor & Wilson [1940]. The dried bacterial bodies and several batches of antigen used in this work were prepared in the Departments of Biochemistry and of Bacteriology, London School of Hygiene and Tropical Medicine; the author is greatly indebted to Professors H. Raistrick and W. W. C. Topley for the use of these starting materials. The antigenic complex was obtained by tryptic digestion [Freeman *et al.* 1940]; the dried organisms were digested in batches of 45 g. with an average yield of 6·9 % of antigen.

Purification of the antigenic complex

Before degradation, the antigenic material was fractionally precipitated from aqueous solution, (*a*) with ethanol and (*b*) with ammonium sulphate, in order to establish its homogeneity.

(*a*) *Ethanol fractionation of* Salmonella typhimurium *antigen.* 23·2 g. of antigen were dispersed in 600 ml. of water by grinding in a mortar and by mechanical shaking. The opalescent viscous solution was made 0·1 N to acetic acid and centrifuged at 3000 r.p.m. for 3 hr., giving 0·24 % of dark insoluble residue. The opalescent supernatant solution gave no appreciable precipitate when ethanol was added to a concentration of 50 % (w/w), but when the ethanol content was raised to 60 % the bulk of the antigen separated out after standing overnight, leaving a water-clear supernatant solution, which contained little or no antigenic material. The precipitate was washed with 60 % ethanol and redispersed in 1 l. of 0·1 N acetic acid. Addition of ethanol to 57 % concentration gave a small amount of precipitate (F 0–60 %/0–57 %), which was removed by passage of the ethanol suspension through a de Laval Industrial Separator (F 0–60 %/0–57 %; 1·25 g. = 5·4 %; N, 5·2; ash, 13·4; reducing sugar value, 58 %; limit of precipitation with antiserum, 1 in 338,000; optimum, 7500–37,500), and a colourless, opalescent supernatant solution. The ethanol content of the latter was raised to 60 % and the bulk of the antigen separated out leaving a clear supernatant solution. The main antigenic fraction, F 0–60 %/57–60 %, was washed twice with absolute ethanol and twice with ether and dried *in vacuo* over P_2O_5 (19·1 g. = 82·4 %; N, 2·7; ash, 14·2; R.V. (reducing sugar value), 65 %; $[\alpha]_D^{18°}$ (in formamide, $c = 1$), $+106° \pm 2°$, limit of precipitation with antiserum 1 in 338,000; optimum, 7500). 50 hr. electrodialysis in cellophane membranes reduced the ash content of F 0–60 %/57–60 % to 7·9 %; N, 2·8 %. The contents of the first and second 60 % ethanol supernatant solutions were practically devoid of antigenic material. Total recovery in fractionation was 97·6 %.

Reducing sugar value (R.V.) and specific rotation determinations on the isolated fractions were carried out as described by Freeman & Anderson [1941], and other analyses were by Dr G. Weiler.

(*b*) *Ammonium sulphate fractionation of antigen.* A further attempt to fractionate the antigenic material was made by means of precipitation from aqueous solution by increasing concentrations of ammonium sulphate. A preliminary experiment showed that the bulk of the antigen was precipitated between the levels of 23–35 % concentration of ammonium sulphate.

The ethanol fractionated antigen (F 0–60 %/57–60 %), 14·9 g. in 500 ml. of water, was three times fractionated between the limits of 23 and 35 % concentration of ammonium sulphate. The isolated fractions were submitted to dialysis to remove the ammonium sulphate, first in collodion sacs, and then to electrodialysis for 60 hr. between cellophane membranes. The bulk of the antigenic material (10·03 g. = 67·5 %) was precipitated at 35 % concentration of ammonium sulphate (F 23–35 %; N, 1·72; R.V. 65; ash, 4·2 %; $[\alpha]_D^{17°}$ (in formamide, $c = 1$), $+95° \pm 2°$; limit of precipitation with antiserum 1 in 1,000,000, optimum 37,500). When the ammonium sulphate concentration was raised to 41 %, a further active fraction (F 35–41 %) was obtained (0·779 g. = 5·3 %, N, 2·5; R.V. 65; ash, 5·8 %; $[\alpha]_D^{17°}$ (in formamide, $c = 1$), $+91° \pm 9°$; limit of precipitation with antiserum 1 in 1,000,000, optimum 37,500). Total recovery in fractionation was 77 %.

Extraction of the antigenic material with organic solvents. Extraction of the antigenic complex with organic solvents [cf. Morgan & Partridge, 1940*a*] yielded only 17 mg. or 0·34 % of fatty fraction. It is therefore apparent that the lipid fraction which was obtained from the antigenic complex on hydro-

lysis in a yield of about 4%, as described later, was an essential antigenic component and not merely an adventitious impurity.

Properties of the antigenic complex

The antigenic complex of *S. typhimurium* gave opalescent aqueous solutions even when the concentration of antigen was as low as 0·4%. The bulk of the substance remained in solution after 30 min. centrifuging at 15,000 r.p.m. The antigenic complex also dissolved readily in formamide and in concentrated formic acid, giving solutions which were practically free from opalescence, but it was insoluble in glacial acetic acid and in dioxan. The bulk of the substance was insoluble in concentrated aqueous phenol solutions but, on exposure to 95% aqueous phenol, the antigenic material swelled up to form a thin translucent jelly which separated out on centrifuging. 0·4% aqueous solutions of the antigen gave no precipitates on addition of an equal volume of uranyl acetate (cf. antigen of *Eberth. typhosa* Ty 2), 10% trichloroacetic acid, salicylsulphonic acid, tannic acid solution, or Esbach's reagent. Phosphotungstic acid (2% in 5% H_2SO_4) gave an immediate flocculent precipitate. The presence of tyrosine residues was indicated by positive Millon's α-nitroso-β-naphthol and Ehrlich's diazo reactions. A weakly positive Sakaguchi reaction for arginine was obtained, but Ehrlich's diazo reaction for histidine was negative. 20 mg. of the substance in 1 ml. of water gave a weakly positive biuret reaction, but the ninhydrin reaction was negative. The substance gave a strong Molisch's reaction but did not reduce Fehling's solution.

On analysis the pure antigen gave C, 48·4; H, 7·3; N, 1·8–1·9; non-volatile ash, 4–5; volatile fatty acid on hydrolysis (calculated as acetyl groups), 6·1%; $[\alpha]_D^{17°}$, +95° ± 1° (in formamide, $c=1$); $[\alpha]_D^{17°}$, +96° (in water, $c=1$); reducing value (method of Hagedorn & Jensen [1923]) on hydrolysis with 2N HCl for 2 hr. in a sealed tube, 65%; organic P, 0·5; inorganic P, 0·3% (N/organic P ratio, 7). Determination of amino-N (Van Slyke) showed that 0·2% of N was present as amino-groups.

Fractionation of antigen from aqueous solution with acetone. To provide further evidence of the homogeneity of the antigenic preparation after purification by fractionation from aqueous solution, first with ethanol and then with ammonium sulphate, the substance was submitted to a further fractionation from aqueous solution with acetone.

Antigen (1·98 g.) was dissolved in 100 ml. water; the bulk of the substance (1·787 g. = 90·2%; N, 1·9; ash, 4·7; R.V. 63%; $[\alpha]_D$ (in formamide, $c=1$), +95° ± 1°; limit of precipitation with antiserum 1 in 1,000,000; optimum, 37,500) was precipitated between acetone concentrations of 43 and 45% (by weight). On raising the acetone concentration to 52% a further fraction (F45–52%) was obtained (0·169 g. = 8·5%; N, 1·8; ash, 4·9; R.V. 65%; $[\alpha]_D$, +97° ± 1°; limit of precipitation with antiserum, 1 in 1,000,000; optimum, 37,500). 0·022 g. (1·1%) of substance was recovered from the supernatant solution.

Immunological properties of Salmonella typhimurium *antigen.* The antigen in its unfractionated state was toxic for mice, average lethal dose, 0·13–0·27 mg. [Freeman *et al.* 1940]. Mouse protection experiments have been carried out on several batches of crude antigen [cf. Raistrick & Topley, 1934; Topley, Raistrick, Wilson, Stacey, Challinor & Clark, 1937]; the material was shown to contain antigens comparable in immunological activity with that of the dried bacterial cells. It has not yet been possible to conduct mouse protection tests on the purified antigen, but this material stimulated the production of precipitins in rabbits at least as readily as the crude substance.

Formamide dissociation of the antigenic complex. Morgan & Partridge [1940*a*] have shown that the antigen of *Bact. dysenteriae* Shiga (a phospholipin-polysaccharide-conjugated protein complex) may be dissociated by the action of neutral formamide with the production of the free phospholipin and a polysaccharide-conjugated protein complex.

The antigen of *S. typhimurium* (1·13 g.) was submitted to dissociation by the method of Morgan & Partridge and yielded only 0·011 g. (0·9%) of a colourless, lipid fraction and 0·894 g. (79%) of 'formamide dissociated antigen'. The latter did not differ significantly in its analytical data (N, 1·8; ash, 5·2%; $[\alpha]_D^{15°}$, +101° ± 1°), limit of precipitation with antiserum and chemical properties, from the undissociated antigenic complex. The aqueous solutions were, however, slightly less opalescent than those of the original antigen, and on addition of NaOH to pH 8·5–10, solutions of the formamide-dissociated product became almost water-clear, whereas those of the antigenic complex were still definitely opalescent.

Acetic acid hydrolysis of the antigenic complex. The antigenic complex was split up into its polysaccharide, lipid and conjugated protein components by hydrolysis with dilute acetic acid [Freeman & Anderson, 1941; Freeman, 1942].

Purified antigenic complex (1·9 g.) was dispersed in 200 ml. of 0·1N acetic acid and, after 2 hr. hydrolysis at 100°, the optical rotation of the supernatant solution had become constant ($[\alpha]_D^{18°}$, +0·69° ± 0·01° (1 dm.)), indicating that hydrolysis was complete.

The following fractions were obtained:

(I) Specific polysaccharide (F0–86%), 1·326 g. = 69·8%; N, nil; ash, 4·9; R.V. 93%; $[\alpha]_D^{20°}$, +105° ± 1° (in water, $c=1$);

(II) Conjugated protein, 0·292 g. = 15·4%; N, 7·2; ash, 5·9%; $[\alpha]_D^{20°}$, −22° ± 4° (in 0·1N NaOH, $c=0·3$); P, 2·1%; N/P ratio, 7; a grey amorphous solid, insoluble in water, but readily soluble in dilute sodium hydroxide and reprecipitated on acidification;

(III) Lipid fraction, 0·074 g. = 3·9 %, a semi-solid, colourless fatty substance;

(IV) An ethanol residue fraction (F 86–99 %), 0·16 g. = 8·4 %; N, 2·7 %; ash, 6·4; R.V. 49 %; $[\alpha]_D^{16°}$, +15° ± 1°.

Fission of the antigenic complex, as described above, is a true hydrolytic breakdown and not merely a thermal disintegration of a colloidal complex. A specimen of antigen underwent no apparent change on heating at 100° in aqueous solution for 2 hr., and gave only 2 % of insoluble residue on centrifuging; on acidification with acetic acid and 2 hr. heating at 100° the supernatant solution broke up into its four components, as described above.

The specific polysaccharide component of the antigenic complex

The crude polysaccharide was purified by electrodialysis to remove the bulk of the ash and submitted to 2–3 fractionations from aqueous solution with ethanol.

In a typical experiment 1·83 g. of crude polysaccharide in 150 ml. water were electrodialyzed for 30 hr. in collodion membranes (Hg cathode, Pt anode, e.m.f. 100 V. d.c.), the solution concentrated *in vacuo* to about 20 ml. volume, treated with 20 mg. of norit for 30 min. at 100° and twice fractionated between the limits of 45 and 86 % ethanol concentration, giving 1·42 g. = 78 % of purified polysaccharide (F 45–86 % (2)). This gave on analysis: C, 44·6; H, 6·9; ash, 1·5 %; acetyl, nil; $[\alpha]_D^{19°}$, +103° ± 1° (in water, $c = 1$). $C_6H_{10}O_5$ requires C, 44·4; H, 6·2 %. A specimen of the electrodialyzed polysaccharide was re-electrodialyzed in 5 % solution for 70 hr., and the polysaccharide, reisolated by addition of ethanol to 86 % concentration, then gave on analysis: C, 44·5; H, 6·9; ash, 1·5 %.

It was concluded that the ash (1·5 %) was combined in the polysaccharide molecule and could not be eliminated by physical means. The pure polysaccharide precipitated with *S. typhimurium* antiserum to a dilution of 1 in 250,000. The polysaccharide gave a strongly positive Molisch's reaction but did not reduce Fehling's solution; it gave no iodine coloration. The absence of fructose residues in the molecule was demonstrated by negative Foulger's and Seliwanoff's tests. Cole's rapid furfural test, Tollens's phloroglucinol test, Bial's orcinol test and the aniline acetate test for furfural, all gave negative results, suggesting the absence of pentose residues. The polysaccharide gave a negative reaction in Tollens's naphthoresorcinol test for uronic acids: a control test on 5 mg. of glucuronic acid under the same conditions gave a strongly positive reaction. Scherer's reaction for inositol and Salkowski's [1910] modification gave negative results on 10 mg. quantities of polysaccharide; 5 mg. of inositol gave strongly positive reactions, both alone and in the presence of 10 mg. of polysaccharide.

Evidence as to the homogeneity of the polysaccharide has been obtained from: (1) attempts to separate it into fractions with different chemical properties by fractional precipitation from aqueous solution with acetone, (2) regeneration of the substance from its triacetyl derivative in an unchanged form, and (3) ultracentrifuge experiments (see Addendum).

Fractionation of polysaccharide from aqueous solution with acetone. 1·49 g. of specific polysaccharide dissolved in 40 ml. of water, giving an almost colourless, water-clear solution, free from undissolved residue, and addition of acetone to a concentration of 40 % by weight gave no opalescence, but when the acetone concentration was raised to 45 % a small amount of polysaccharide was precipitated. This was separated off, triturated with ethanol, washed twice with ether, and dried *in vacuo* over phosphorus pentoxide. Further increments of acetone were added until the material had been divided into seven fractions: F 40–45 %, F 45–50 %, F 50–55 %, F 55–60 %, F 60–65 %, F 65–75 %, and F 75–99 %. The bulk of the substance (77·6 %) was precipitated between acetone concentrations of 40 and 55 %, and only 6·2 % remained soluble in 65 % acetone. The seven fractions were compared by determination of specific optical rotation and reducing value on hydrolysis (Table 1).

Table 1. *Acetone fractionation of specific polysaccharide of* S. typhimurium

Fraction	Weight (g.)	Yield (%)	$[\alpha]_D^{20°}$ (in water, $c = 1$)	Reducing value on hydrolysis (percentage of that of glucose)
Unfractionated polysaccharide	1·4902	—	+103°	93
(1) F 40–45 %	0·1666	11·2	+101°	85
(2) F 45–50 %	0·6770	45·4	+101°	90
(3) F 50–55 %	0·3131	21·0	+101°	86
(4) F 55–60 %	0·0774	5·2	+101°	88
(5) F 60–65 %	0·0944	6·3	+105°	84
(6) F 65–75 %	0·0602	4·0	—	66
(7) F 75–99 %	0·0331	2·2	—	74

Total recovery: 95·3 % (results uncorrected for ash).

Fractions of the polysaccharide differing in solubility in acetone-water mixtures have been shown by specific rotation and reducing value determinations to be practically identical, and it may be concluded that the specific polysaccharide represents a chemical entity.

Acetylation of polysaccharide. On acetylation with acetic anhydride in pyridine solution at 70° for 1 hr., the substance (0·58 g.) yielded 65 % of theory (0·68 g.) of crude acetyl polysaccharide. The latter was purified by extraction with boiling absolute ethanol and by reprecipitation from chloroform solution with light petroleum. The two fractions precipitated by light petroleum concentrations of 63 and 85 % respectively were found to be identical on analysis, showing that the acetyl compound was homogeneous. The pure acetyl polysaccharide (triacetyl derivative) was a

colourless amorphous solid; it was insoluble, even on boiling, in absolute ethanol, methanol, ether and petroleum ether, but was readily soluble in chloroform, acetone, pyridine and acetic anhydride. It was strongly dextrorotatory, $[\alpha]_D^{18°}$, $+113° \pm 1°$ (in chloroform, $c=1$), and gave on analysis: C, 49·9; H, 6·2; acetyl, 48·8%. $C_6H_7O_5(OC.CH_3)_3$ requires C, 50·0; H, 5·6; CH_3CO, 44·8%.

Regeneration of the polysaccharide from its acetyl derivative. The acetyl derivative was deacetylated under the conditions worked out for the deacetylation of the *Eberth. typhosa* triacetyl polysaccharide [Freeman, 1942]. 0·48 g. of the acetyl polysaccharide gave 0·228 g. (84·4% of theory) of specific polysaccharide, which gave on analysis: $[\alpha]_D^{18°}$, $+102° \pm 1°$ (in water, $c=1$); C, 46·3; H, 6·85; acetyl, 0·9; ash, 0·7%, and precipitated to a dilution of 1 in 300,000 with *S. typhimurium* antiserum, optimum 1 in 150,000.

Acid hydrolysis of the specific polysaccharide. The optimal conditions of liberation of reducing sugars by hydrolysis of the polysaccharide were investigated as a preliminary step in the identification of the sugars present. The hydrolysis in N H_2SO_4 at 100° proceeds rapidly to about 87% (2 hr.) [Hagedorn & Jensen] and then follows a slower linear increase, reaching 93% hydrolysis after 12 hr. The unhydrolyzed polysaccharide had a reducing value, as glucose, of 1·3%. It was found that for complete hydrolysis of the polysaccharide 7 hr. heating with N H_2SO_4 at 100° was adequate.

Nature of the component reducing sugars

The specific polysaccharide has been shown to consist predominantly of anhydrohexose units; ketose, pentose and uronic acid residues were absent. *d*-Galactose has been shown to be present in the antigenic complex from which the polysaccharide was derived [Freeman *et al.* 1940].

The hydrolysate from 0·971 g. of specific polysaccharide, freed from H_2SO_4 and concentrated to 25 ml., gave $\alpha_D^{19°}$, $+1·12°$ (1 dm.); total apparent glucose = 0·8115 g. (83·6% of the polysaccharide). If the reducing constituents had a reducing value equal to that of glucose, the 'apparent specific rotation' of the mixture was $[\alpha]_D^{19°}$, $+34·5°$.

d-*Mannose-phenylhydrazone.* One-half of the total neutral hydrolysate was treated with 0·2 g. phenylhydrazine and 0·4 g. 25% acetic acid [van der Haar, 1920; Freeman, 1942]; mannose phenylhydrazone crystallized out on standing at 0°, yield 0·1486 g., corresponding to a mannose content of 20·4% of the specific polysaccharide. The crude product was recrystallized from boiling aqueous ethanol (60% w/w) and gave *d*-mannose phenylhydrazone, colourless microcrystalline plates, m.p. 195° (186° on slow heating), unchanged on admixture with an authentic specimen. Found: C, 53·2; H, 6·7; N, 10·3%. $C_{12}H_{18}N_2O_5$ requires C, 53·3; H, 6·7; N, 10·4%.

d-*Galactose.* The excess phenylhydrazine was removed as benzaldehyde phenylhydrazone [Morgan, 1938], the filtrate concentrated *in vacuo* to dryness and the residue of sugars taken up in 2 ml. 90% acetic acid and the solution allowed to crystallize at 0°. 0·053 g. of crystalline *d*-galactose (10·8% of the polysaccharide) separated out. The product reduced Fehling's solution (m.p. 161°, mixed m.p. with authentic *d*-galactose (m.p. 164°) was 161°, $[\alpha]_D^{19°}$, $+80° \pm 2°$ (in water, $c=1$)). A specimen (0·038 g.) yielded 90% (0·054 g.) of *d*-galactose methylphenylhydrazone, m.p. 190°, unchanged on admixture with an authentic specimen.

A small further yield of *d*-galactose (2·0% of the polysaccharide) as the methylphenylhydrazone was obtained from the filtrate after removal of the crystalline *d*-galactose by treatment with methylphenylhydrazine (asym.) [Neuberg, 1907; Lüdtke, 1929; Freeman, 1942]. Found: C, 55·0; H, 7·1; N, 10·0%. $C_{13}H_{20}N_2O_5$ requires C, 54·9; H, 7·1; N, 9·9%.

d-*Glucose*-p-*nitrophenylhydrazone.* Excess methylphenylhydrazine was eliminated as described above, the sugar-containing filtrate evaporated to dryness *in vacuo* and the residue taken up in 2 ml. absolute methanol and treated with *p*-nitrophenylhydrazine (0·1 g.) [van Ekenstein & Blanksma, 1903; Reclaire, 1908; Freeman, 1942]. Yellow crystalline *d*-glucose-*p*-nitrophenylhydrazone was obtained (0·044 g., 5·2% of glucose), m.p. 184°, unchanged on admixture with authentic hydrazone. Found: C, 45·7; H, 5·6; N, 13·3%. $C_{12}H_{17}O_7N_3$ requires C, 45·7; H, 5·4; N, 13·3%.

The isolations of sugars, as substituted phenylhydrazones, under conditions in which the derivative of a single hexose is obtained at each stage, provide conclusive proof of the co-existence of *d*-glucose, *d*-mannose and *d*-galactose as constituents of the specific polysaccharide. The losses of sugars in the intermediate removal of excess of substituted hydrazines with benzaldehyde are, however, sufficiently great to lower significantly the yields of hydrazones.

In a further series of experiments the neutralized hydrolysate from 1·45 g. of polysaccharide gave 0·274 g. (18·9%) of crystalline *d*-galactose. After recrystallization from 90% acetic acid, the m.p. was 165°, unchanged on admixture with authentic *d*-galactose; the initial and equilibrium rotations (in water, $c=1·7$) were: $[\alpha]_D^{15°}$, $+143° \pm 1°$ (after 3 min.); $+80·5° \pm 1°$ (after 24 hr.) (these agree closely with the recorded values for α-*d*-galactose, $+144·0° \rightarrow +80·5°$ [Hudson & Yanovsky, 1917]) and $+80·4°$ (at equilibrium) [Parcus & Tollens, 1890].

The residual sugars in solution were heated under reflux with *p*-nitrophenylhydrazine (1 g.) in methanol. *d*-Mannose-*p*-nitrophenylhydrazone (0·473 g., 18·6% mannose in terms of the polysaccharide) crystallized out as pale yellowish brown crystals from the boiling solution, m.p. 190°, unchanged on admixture with authentic *d*-mannose-*p*-nitrophenylhydrazone. On admixture with *d*-glucose-*p*-nitrophenylhydrazone (m.p. 184°) the mixed m.p. was 175°. $[\alpha]_D^{14°}$, $+86° \pm 1°$ (in pyridine, $c=1$), which agreed with that of the authentic hydrazone ($[\alpha]_D^{15°}$, $+87° \pm 1°$).

0·6234 g. (24·6% as *d*-glucose) of *d*-glucose-*p*-nitrophenylhydrazone separated out from the filtrate at 0° after removal of *d*-mannose-*p*-nitrophenylhydrazone.

Thus 18·9% of *d*-galactose has been isolated from the specific polysaccharide as the free sugar, in addition to yields of substituted hydrazones equivalent to 24·6% *d*-glucose, and to 20·4% *d*-mannose (20·4% as the phenylhydrazone, and in another experiment 18·6% as the *p*-nitrophenylhydrazone). Control experiments under the same conditions with similar concentrations of single pure hexoses have

shown that pure *d*-glucose gives 79·6% of the theoretical yield of the *p*-nitrophenylhydrazone and pure mannose 94·9% of the theoretical yield of *d*-mannose phenylhydrazone [Freeman, 1942]. On this basis the actual hexose contents of the specific polysaccharide are estimated to be: *d*-glucose, 31; *d*-mannose, 21·5; *d*-galactose, 18·9; total hexose, 71·4%. The latter approaches reasonably close to the reducing value of 90% (as apparent glucose) on hydrolysis of the polysaccharide.

A useful confirmation of the relative proportions of the three hexoses present in the polysaccharide hydrolysates is afforded by a comparison of the observed optical rotation of a neutralized hydrolysate with that calculated from the reducing value of the solution (Hagedorn & Jensen) in terms of apparent glucose. The hydrolysate described above had $\alpha_D^{18\cdot5^\circ}$, +1·12° (1 dm.), total volume 25 ml. and total apparent glucose, 0·8115 g. In such a solution, containing 31% glucose, 21·5% mannose and 18·9% galactose, the rotation of the *d*-glucose component is +0·53°, that of the mannose component +0·10° and that of the galactose +0·50°, giving a total calculated rotation (1 dm.) of +1·13°, which agrees closely with the observed value.

The conjugated protein component of the antigenic complex

The conjugated protein fraction gave positive biuret, Millon's and Sakaguchi's reactions, showing the presence of tyrosine and arginine residues. Ehrlich's diazo reaction for histidine was also positive.

The crude substance (0·63 g.) was purified by three precipitations from 0·1N NaOH with 4N HCl [Freeman & Anderson, 1941] and gave 76% (0·48 g.) of purified conjugated protein, N, 6·2; ash, 5·1%.

Tryptic digestion of the conjugated protein. 0·0725 g. of the conjugated protein was freshly precipitated by acidifying an alkaline solution of the substance, and suspended in 15 ml. of borate buffer, pH 8·4; trypsin (0·003 g.) was added and the digest incubated at 37°. After 5 days' digestion only 5·1% of the total N had been liberated as amino-N, and 17·7% after 14 days (Van Slyke). The substance was, therefore, only slowly and very incompletely attacked by trypsin.

Acid hydrolysis of the conjugated protein. 0·01 g. quantities of the conjugated protein were hydrolyzed with 1 ml. 10N formic acid containing 3·6% HCl in sealed tubes at 100° for various periods of time and the amino-N liberated determined by the Van Slyke method [Morgan & Partridge, 1940*a*; Freeman & Anderson, 1941]. After 2–4 hr. hydrolysis the bulk of the conjugated protein had dissolved, giving a pale brown solution, although traces of insoluble residue were still present after 8 hr. heating. The amino-N, liberated from the conjugated protein, progressively increased to 50·5% of the total N after 4–8 hr. hydrolysis and at this stage liberation of amino-acids had reached a maximum.

The 86% ethanol-soluble fraction of the antigenic complex. In its crude state this fraction was a colourless, very hygroscopic solid, which dissolved readily in water but was insoluble in chloroform and acetone; it gave a strongly positive Molisch's reaction, showing the presence of carbohydrate residues, but negative ninhydrin and biuret reactions, from which it was concluded that the nitrogen was not present as amino-acids or peptides. The fraction reduced Fehling's solution on being boiled with it for about 20 sec. It gave an immediate precipitate with 2% phosphotungstic acid in 5% H_2SO_4. On hydrolysis the substance yielded 21·5% of volatile fatty acid (calculated as acetyl) and a trace of glucosamine (1%). The nature of this fraction and its role in the antigenic complex are not yet understood. The compound may be a partially acetylated polysaccharide. The bulk of the volatile fatty acid component (acetyl) of the antigenic complex is present in the 86% ethanol-soluble fraction. The substance has no power of specific precipitation with *S. typhimurium* antiserum.

The lipid components of the antigenic complex

The lipid fraction liberated on acetic acid hydrolysis constitutes only 3–4% of the antigen. By dissociation of the antigenic complex with formamide a part of this lipid fraction (0·9% of the antigen) is liberated. Although other workers have isolated much larger amounts of lipid components from bacterial antigens [Morgan & Partridge, 1940*a*; Soru & Combiesco, 1940; Boivin & Mesrobeanu, 1938], our *S. typhimurium* antigen does not appear to contain more than about 5% of this component.

0·145 g. of the lipid fraction was separated by the usual methods, into a mixed fatty acid fraction (colourless solid, 0·063 g., 43%), a fraction insoluble in cold acetone, which was probably a phospholipin (0·09 g. = 6·4%), and 0·04 g. (28%) of neutral fatty substance. Insufficient of the lipid component was available for further investigation.

Preparation of the specific polysaccharide directly from the dried bacterial bodies

As in the case of *Eberth. typhosa* Ty 2, the specific polysaccharide of *S. typhimurium* may be extracted directly from the dried bacterial cells by means of dilute acetic acid at 100° as described by Freeman [1942] [cf. White, 1929, and Morgan, 1936].

28 g. of the dried cells were extracted three times for 1½ hr. at 100° with 400 ml. lots of 0·1N acetic acid. The successive extracts had optical rotations of +0·57°, +0·12° and −0·06° (1 dm., 18°), and yielded 4·3 g. (15·4%) of colourless crude polysaccharide (N, 5·9; ash, 25·9; R.V. 84%; $[\alpha]_D^{16^\circ}$, +67°±4° (in water, $c=1$)). The substance precipitated with *S. typhimurium* antiserum to a dilution of 1 in 300,000.

The polysaccharide was purified as described by Freeman [1942]; 1·88 g. of the crude material gave 0·564 g. (30%) of pure polysaccharide, which gave on analysis: C, 45·4; H, 6·8; inorganic P, 0·1%; organic P, 0·3%; N, nil. The yield of pure polysaccharide was 4·6% of the dried bacterial bodies, as compared with a yield of about 4·8% in terms of the dried bacterial bodies, by hydrolysis of the isolated antigenic complex.

A rough strain of *S. typhimurium* (100 g. dried bodies) was twice extracted with 1000 ml. lots of 0·1N acetic acid for 1½ hr. at 100°. The extracts, which had optical rotations of +0·15° and +0·06° (1 dm., 17°), were found to contain no significant amount of the specific polysaccharide. A fraction F50–86% (1) was obtained (4·2%), which gave only a trace of precipitate with *S. typhimurium* antiserum at a dilution of 1 in 15,000. The specific polysaccharide tested at the same time precipitated to a dilution of 1 in 300,000.

Preparation of an undegraded specific polysaccharide

Morgan & Partridge [1941] have shown that by dissociation of the antigenic complex of *Bact. dysenteriae* (Shiga) with dilute NaOH, and by means of extraction with concentrated aqueous solutions of phenol, a specific non-antigenic polysaccharide was obtained, which differed markedly, in chemical and physical properties, from the ('degraded') polysaccharide obtained by means of hydrolysis with hot dilute acetic acid, and possessed the power, not shared by the 'degraded' polysaccharide, of recombining with the conjugated protein component of the antigen to produce antigenically active complexes. The antigenic complex of *S. typhimurium* has been submitted to fractional degradation by methods analogous to those described by Morgan & Partridge.

(1) *By sodium hydroxide dissociation.* The *S. typhimurium* antigen (1·34 g.), in 100 ml. of water, was treated with NaOH to a concentration of 0·05N at 0°, as described by Morgan & Partridge; 1·13 g. (85%) of 'recovered antigen' was obtained, and this did not differ significantly from the untreated antigen in its solubility, appearance in solution and qualitative reactions. Only 0·007 g. (0·5%) of material was recovered from the alkaline alcoholic supernatant solution. This gave a negative biuret reaction. It was clear that 0·05N sodium hydroxide had no significant dissociating effect on the antigenic complex.

'Recovered antigen' from the previous experiment was treated with 0·25N NaOH. An ethanol precipitated fraction (F68% (2), 1·01 g. = 94%; N, 1·2; ash, 2·2%; $[\alpha]_D^{18°}$, +104° ±1° (in formamide, $c = 1$)) and a protein-like fraction soluble in alkaline ethanol, were obtained. The latter (0·041 g. = 3·9%; N, 9·0%; ash, nil) was a colourless, amorphous solid, which was insoluble in water but dissolved freely in 0·1N NaOH and was slowly precipitated from alkaline solution on acidification with 2N HCl. The substance gave positive biuret, Sakaguchi's and Ehrlich's diazo reactions, indicating the presence of peptides and arginine and histidine residues. The compound was amphoteric; it dissolved slowly in N acetic acid at 40° as well as in dilute alkali. After reprecipitation from alkaline solution and from solution in N acetic acid, the substance remained virtually unchanged, N = 9·3%.

The recovered 'polysaccharide' (F68% (2), 0·91 g.) was submitted to two further dissociations with 0·25N alkali and gave 0·68 g. (75%) of 'undegraded polysaccharide' (F68% (4), N, 0·5%), equivalent to 60% of the undissociated antigen. The undegraded polysaccharide gave aqueous solutions very much less opalescent than those of the original antigenic complex. 0·021 g. (2·3%) of amphoteric protein fraction was also obtained from the third and fourth alkali treatments of the antigen.

In order to establish the homogeneity of the undegraded polysaccharide, F68% (4) (0·62 g.) was fractionally precipitated from aqueous solution with ethanol. Three fractions were isolated:

(*a*) F55–60% (0·56 g. = 90·9%; C, 47·1; H, 7·3; acetyl 3·5; ash, 6·3; R.V. 73%; $[\alpha]_D^{16°}$, +112° ±1°; limit of precipitation with antiserum, 1 in 300,000, optimum 150,000),

(*b*) F60–70% (0·004 g. = 0·7%), and

(*c*) F70–99% (0·048 g. = 7·8%; R.V. 75%; limit of precipitation with antiserum 1 in 150,000; optimum 1500),

and it was concluded from the results that the alkali-dissociated polysaccharide, F68% (4), was chemically homogeneous. Further evidence on the homogeneity of this substance was provided by ultracentrifugal sedimentation (see Addendum).

(2) *By extraction of the antigenic complex with concentrated aqueous phenol solutions. Salmonella typhimurium* antigenic complex (0·984 g.) was repeatedly extracted with 80% aqueous phenol and 95% aqueous phenol; the former yielded 0·027 g. (2·7%) of amphoteric protein and the latter none. The 'recovered polysaccharide' (1) (0·75 g. = 76·5%; $[\alpha]_D^{18°}$, +81° ±3° (in formamide, $c = 1$)) was an amorphous substance which gave very opalescent aqueous solutions. Recovered polysaccharide (1) (0·722 g.) was three times re-extracted with 80% phenol and gave 0·621 g. (86·0%) of recovered polysaccharide (2) (N, 0·5%). The latter gave very opalescent aqueous solutions similar to those of the original antigen, and was precipitated from solution by 2% phosphotungstic acid in 5% H_2SO_4.

In order to investigate the homogeneity of recovered polysaccharide (2), the material (0·559 g.) was fractionally precipitated from aqueous solution (50 ml.) with ethanol. The properties of the fractions isolated are summarized in Table 2. Fraction F50–60% gave on analysis, C, 49·1; H, 7·9; ash, 5·5%.

Table 2. *Analytical data for fractions of* S. typhimurium *'undegraded polysaccharide' prepared by phenolic extraction*

	Insoluble residue	Ethanol fractions of 'polysaccharide'		
		F50–60%	F60–65%	F65–99%
Weight (g.)	0·069	0·298	0·112	0·019
Yield (%)	12·3	53·4	20·1	3·5
N (%)	—	0·9	—	—
Reducing value on hydrolysis (percentage of that of glucose)	—	66	72	—
Dilution limit of precipitation with *S. typhimurium* antiserum (1 part dissolved in:)	—	1,000,000	1,000,000	—
Dilution giving optimal precipitation (1 part dissolved in:)	—	300,000	300,000	—

Total recovery: 89·3%.

Recombination of Salmonella typhimurium *'undegraded polysaccharides' with proteins*

Antigenically active artificial complexes of the specific polysaccharide and conjugated protein components of *Bact. dysenteriae* (Shiga) have been obtained by Morgan & Partridge [1940 *a*, *b*] by dissolving the isolated components in formamide and precipitating the complex with ethanol, and later, using an undegraded specific polysaccharide, simply by mixing the components of the complex in alkaline solution [Morgan & Partridge, 1941]. The observation that bacterial polysaccharides possess the power of forming acid-soluble complexes with proteins, which are normally insoluble in aqueous solution at acid reaction, has been confirmed and extended to the undegraded polysaccharide of *S. typhimurium*. If a solution of the undegraded polysaccharide and an alkaline solution of the protein are mixed and acidified with acetic acid, a soluble, stable complex is formed, and the protein is no longer precipitated by the addition of trichloroacetic acid or other protein precipitants, provided that an excess of the protein above that equivalent to the combining power of the undegraded polysaccharide has not been used.

By this means the 'undegraded polysaccharides' prepared by alkaline dissociation and by phenolic extraction of the *S. typhimurium* antigenic complex were found to form acid-soluble complexes with the conjugated proteins of *S. typhimurium* and *Eberth. typhosa* Ty2. When the specific polysaccharide (acetic acid hydrolytic product) replaced the undegraded polysaccharide, the admixed proteins were immediately precipitated by acid. The alkali-dissociated undegraded polysaccharide had the power of rendering soluble at least an equal weight of conjugated protein (*S. typhimurium*), but the phenol-extracted 'polysaccharide' was saturated by more than 40% of protein; this shows that the two 'undegraded polysaccharides' are clearly different.

The alkali-dissociated undegraded polysaccharide formed trichloroacetic acid-soluble complexes on admixture in alkaline solution with edestin, human serum pseudoglobulin, casein, gliadin and rabbit myosin. When the polysaccharide was mixed with rabbit myosin in neutral salt solution, no acid-soluble complex was formed. Control experiments with *S. typhimurium* conjugated protein and with mixtures of the above proteins with degraded polysaccharide, gum arabic or soluble starch, all gave negative results.

The undegraded polysaccharide lost its power of combining with the conjugated protein after 1 hr. heating in 0·1N acetic acid solution at 100°, but there was no appreciable loss of this activity after 30 min. at 50°, or 5 min. at 100°, in acetic acid. After 1 hr. hydrolysis at 100° with 0·1N acetic acid the undegraded polysaccharide solution had lost its opalescence and had deposited a trace of precipitate (2·1%). At this stage the hydrolysate contained two polysaccharides, which were separated by fractional precipitation with ethanol: (*a*) (F0–86%), 80·2%, $[\alpha]_D^{19°}$, $+100° \pm 1°$; this substance was identical with the degraded polysaccharide prepared by acetic acid hydrolysis of the antigenic complex; and (*b*) (F 86–99%), 0·032 g. = 10·6%; acetyl, 13·2%; insufficient of this component was obtained for adequate purification, but the evidence available strongly suggests that the substance, which was a very hygroscopic acetyl polysaccharide, consisted predominantly of the acetyl polysaccharide previously isolated directly from the antigenic complex.

An artificial complex of the undegraded polysaccharide and the conjugated protein behaved, on heating at 100° in 0·1N acetic acid solution, in a manner identical with that of the native antigenic complex. An artificial complex of undegraded polysaccharide and horse serum albumin was stable in neutral solution at 100° for 2 hr., giving no precipitation of the albumin.

Combination of undegraded polysaccharide and horse serum albumin. Determination of saturation value. The undegraded polysaccharide was found to form a trichloroacetic acid-soluble complex with horse serum albumin when the two components were mixed either in neutral or in alkaline solution at 30°. (The horse serum albumin was a specimen of lyophil-dried protein kindly supplied by Dr A. G. Ogston; see Addendum.) When the ratio protein/polysaccharide did not exceed 3·5, trichloroacetic acid-soluble complexes were formed, but increase of protein above this proportion resulted in heavy precipitates on addition of trichloroacetic acid. In the neighbourhood of the equivalence zone addition of the precipitant produced very opalescent solutions, from which a small amount of protein separated out on standing.

The nitrogen contents of the precipitates and supernatant solutions obtained from several undegraded polysaccharide-albumin mixtures, by treatment with 20% trichloroacetic acid, were determined after drying by the Dumas method. It was found that when the ratio protein/polysaccharide did not exceed 3·5, practically the whole of the protein remained in the supernatant solution, but that when this figure was exceeded the whole of the protein was precipitated, practically none remaining in the supernatant solution.

Titration curves of artificial polysaccharide-protein complexes

In order to investigate the changes which take place in the solubility and buffering power of proteins in the presence of *S. typhimurium* undegraded polysaccharide, (*a*) horse serum albumin and

(*b*) *S. typhimurium* conjugated protein, alone and in the presence of the undegraded polysaccharide, were titrated at 19° with 0·01N HCl and NaOH, and the pH determined at intervals with a glass electrode and valve electrometer pH meter.

The following solutions were titrated:

(i) 5 ml. 1% aqueous albumin + 4 ml. water.
(ii) 5 ml. 1% aqueous albumin + 4 ml. 0·5% polysaccharide.
(iii) 5 ml. 1% conjugated protein + 4 ml. water.
(iv) 5 ml. 1% conjugated protein + 4 ml. 0·5% polysaccharide.
(v) 5 ml. water + 4 ml. 0·5% polysaccharide.
(vi) 9 ml. distilled water.

The conjugated protein solution was prepared by dissolving the substance (0·14 g.) in 2·5 ml. 0·1N NaOH, adding 1 ml. 0·1N HCl to the solution and diluting to 14 ml. with water.

Between pH 5·8 and 4·5 in titration (iii) each successive addition of acid produced a slight precipitate, which rapidly redissolved on stirring; at pH 4·47 a permanent precipitate of conjugated protein appeared which separated out on standing, leaving a clear supernatant solution. In the presence of the undegraded polysaccharide (titration (iv)) there was no change in the appearance of the solution and no precipitation of protein.

The undegraded polysaccharide had no appreciable buffering power; titrations (v) and (vi) gave practically identical results. The presence of the undegraded polysaccharide brought about no significant change in the buffering power of serum albumin (Fig. 1) on the acid side of pH 6, but in the range of pH 6–10 there was an appreciable increase of buffering power. The displacement of the serum albumin curve in the presence of the undegraded polysaccharide occurred in the same pH range and in the same sense as that produced by formaldehyde [Prideaux & Woods, 1933], but was not nearly as great. In the experiments of Prideaux & Woods, 5–6 ml. more 0·1N alkali were required to bring the albumin to pH 9 in the presence of formaldehyde, whereas only 12 ml. more 0·01N alkali would be required to bring a corresponding quantity of albumin to pH 9 in the presence of undegraded polysaccharide. In the titration curves of *S. typhimurium* conjugated protein (Fig. 2) there was a marked decrease of buffering power, in the presence of the polysaccharide, in the pH range 4–7, which coincided with the range of solubility of the conjugated protein on the acid side of neutrality.

Katchalsky [1941] observed depression of the pH when glucose was added to solutions of various amino-acids and peptides, from which he has determined the equilibrium constants of the interaction of the two components.

It is clear that interaction or combination takes place between the protein and undegraded polysaccharide molecules, with the production of complexes which behave towards acids and alkalis differently from the untreated protein. Nothing is known of the nature of the protein-polysaccharide bond, but this may be salt-like, or possibly of the Schiff's base type through a polysaccharide alde-

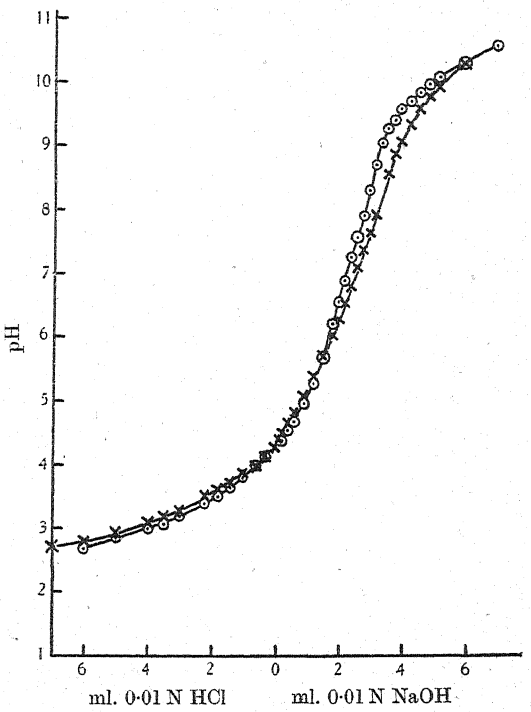

Fig. 1. Titration curve of serum albumin in presence and absence of *Salmonella typhimurium* undegraded polysaccharide. ⊙—⊙ Serum albumin, ×—× serum albumin + polysaccharide.

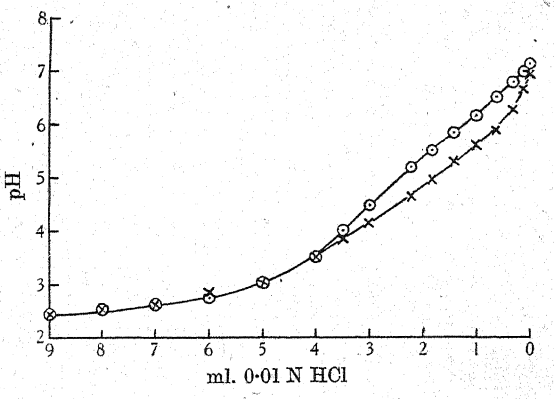

Fig. 2. Titration curve of *Salmonella typhimurium* conjugated protein in presence and absence of undegraded polysaccharide. ⊙—⊙ Conjugated protein, ×—× conjugated protein + polysaccharide.

hydic group and an amino-group in the protein. The evidence available suggests that the combination is not analogous to the interaction of formaldehyde with amino-groups, which involves a considerable pH shift and alteration of the pK's of the amino-groups, whereas in the formation of the undegraded polysaccharide-protein complex there is a very small pH shift, and the pK's of the amino-groups are not seriously changed despite the formation of an apparently stable complex, in which the polysaccharide renders the protein more soluble in the presence of acid precipitants. These conclusions regarding the existence of an artificial protein-polysaccharide complex are confirmed by ultracentrifugal determination of sedimentation constants (see Addendum).

Antigenicity of undegraded polysaccharides and an artificial polysaccharide-conjugated protein complex

The following fractions were tested for antigenicity in rabbits:

(i) Undegraded polysaccharide (phenol-dissociated product).

(ii) Undegraded polysaccharide (alkali-dissociated product).

(iii) 'Degraded' specific polysaccharide.

(iv) *Salmonella typhimurium* conjugated protein (dissolved in phosphate buffer (pH 8·4) plus saline).

(v) Artificial polysaccharide-conjugated protein complex (70 mg. undegraded polysaccharide, alkali dissociated, +20 mg. conjugated protein).

(vi) *Salmonella typhimurium* antigenic complex. (The author is indebted to Dr A. R. Martin for the rabbit immunizations.) The rabbit antisera were tested for the presence of specific precipitins against solutions of the homologous antigens, and for their power of agglutinating killed O-suspensions of *Salmonella typhimurium* (Table 3).

The phenolic-dissociation product and the artificial polysaccharide-conjugated protein complex were powerfully antigenic, and gave rise to antisera which precipitated to high dilutions with the homologous antigens, and agglutinated *S. typhimurium* suspensions to about the same dilution as an antiserum prepared against the purified antigenic complex. The alkali-dissociation product (undegraded polysaccharide) and the degraded specific polysaccharide, on the other hand, had no detectable antigenic activity. The phenolic- and alkali-dissociated products, and the degraded polysaccharide, precipitated with an antiserum prepared against the original antigenic complex to dilutions of 1 in 1,000,000 (optimum 1 in 150,000), 1 in 300,000 (optimum 1 in 15,000) and 1 in 300,000 respectively. *S. typhimurium* conjugated protein was feebly antigenic, giving rise to homologous precipitins, but sera 296 A and B were free from *S. typhimurium* agglutinins and gave no precipitates with the original antigenic complex. By combination of the inactive undegraded polysaccharide and the feebly antigenic conjugated protein, which had no *S. typhimurium* specificity, a powerfully antigenic artificial complex was obtained, which gave rise to antisera which precipitated the homologous antigen and the original antigenic complex (to dilutions of 1 in 600,000, optimum 1 in 15,000) and agglutinated *S. typhimurium* suspensions.

The phenolic-dissociated undegraded polysaccharide precipitated with antisera prepared against the parent antigenic complex in a manner almost identical with that of the alkali-dissociated undegraded polysaccharide, had similar chemical and physical properties and also possessed the power of recombining with the conjugated protein com-

Table 3. *Antigenicity of fractions from* S. typhimurium

Material used as antigen	Rabbit no.	Precipitation test. Dilution of homologous antigen 1 in 1500	15,000	150,000	300,000	Agglutination test. Dilution of immune serum 1 in 40	80	160	320	640	1280
(i) Undegraded polysaccharide (phenol-dissociated product)	263A	+ +	+ + + +	+ + +	+ + +	4	4	4	3	1	0
	263B	+ +	+ + + (optimum)	+ + +	+	4	4	4	3	1	0
(ii) Undegraded polysaccharide (alkali-dissociated product)	266A	—	—	—	—	0	0	0	0	0	0
	266B	—	—	—	—	0	0	0	0	0	0
(iii) 'Degraded' specific polysaccharide	265A	—	—	—	—	0	0	0	0	0	0
	265B	—	—	—	—	0	0	0	0	0	0
(iv) *S. typhimurium* conjugated protein	296A	+ +	+ +	+ +	—	0	0	0	0	0	0
	296B	+ + (optimum)	+ +	+ +	—	0	0	0	0	0	0
(v) Artificial polysaccharide-conjugated protein complex	298A	+ +	+ + +	+	—	4	4	4	3	1	0
	298B	+ + +	+ + + + (optimum)	+ +	+	4	4	4	2	1	0
(vi) *S. typhimurium* antigenic complex	230	+	+ + + (optimum)	+ + +	+ +	4	4	4	4	2	0

4, complete agglutination; 3, almost complete agglutination; 2, incomplete agglutination; 1, trace of agglutination; 0, no agglutination.

ponent, though not so powerfully as the alkali dissociation product. It was clearly different from the latter, as shown by its antigenic properties in rabbits, though this property may be traceable to residual small amounts of the antigenic complex still undecomposed by repeated phenolic extractions.

DISCUSSION

In its nature and properties the antigen of *S. typhimurium* shows a close similarity to the corresponding complex from *Eberth. typhosa* Ty 2. Both antigens exist as polysaccharide-conjugated protein-lipid complexes, which are readily broken down into their components by hydrolysis. There is also a fourth component which has not yet been isolated in a pure condition, but is described provisionally as a partially acetylated polysaccharide. When the antigenic complex is degraded, its antigenic properties are lost but the polysaccharide component retains the specific properties of the complex. The latter undergoes partial degradation, with the elimination of one or more of its components, on treatment with formamide, dilute alkali and concentrated aqueous phenol. Dilute alkali and phenol liberate an amphoteric protein, which differs from the conjugated protein in being soluble at acid reactions, and an 'undegraded polysaccharide', intermediate in properties between the antigenic complex and the specific polysaccharide. The undegraded polysaccharide differs very significantly from the specific polysaccharide, in possessing the power of combining with proteins to form stable complexes, and there is evidence that it consists of a complex of the specific polysaccharide and the acetyl polysaccharide.

Previous workers have isolated antigenic complexes from *S. typhimurium* but some of the earlier preparations were undoubtedly contaminated with materials derived from the culture media and evidence of their chemical homogeneity is not always available. Raistrick & Topley [1934] prepared antigenic fractions which contained 4–6·5 % nitrogen and gave 23·1–37·8 % reducing sugars on hydrolysis. Topley *et al.* [1937] describe an antigenic fraction which contained 4·2–4·8 % N. Boivin's antigen from the same organism contained 1·83–2·20 % N; R.V. 40 % on hydrolysis [Boivin & Mesrobeanu, 1934]. At an earlier stage of this investigation, Freeman *et al.* [1940] obtained the antigen for the first time from organisms grown on a synthetic medium; it contained N, 4·2 %, and gave 50 % of reducing sugars on hydrolysis, $[\alpha]_{5461}$, +76°. These data relate to the crude fraction which was the starting material in the ethanol and ammonium sulphate fractionations in the present work. These figures illustrate the difficulties of obtaining pure antigenic fractions from the bacterial cell, and emphasize the need for careful fractionation of the primary extraction products.

In their behaviour on hydrolysis, and in the properties of their conjugated protein and specific polysaccharide components, the antigenic complexes of *S. typhimurium* and *Eberth. typhosa* display a marked similarity, but there are considerable quantitative differences of nitrogen content, reducing value on hydrolysis and specific optical rotation. The conjugated proteins of the two antigenic complexes are markedly similar in their physical properties, N contents, constituent amino-acids and liberation of amino-nitrogen on tryptic digestion and acidic hydrolysis.

As was to be expected from the immunological data for the two organisms, cross precipitation reactions between the specific polysaccharides of *S. typhimurium* and *Eberth. typhosa* Ty 2 were negative; the chemical data confirm that the two substances are different. Both polysaccharides, however, consist entirely of the same hexose units (*d*-glucose, *d*-mannose and *d*-galactose), which are present in approximately the same relative amounts. Differences in the structural arrangement of the constituent sugars, which may account for the serological dissimilarity of the two specific polysaccharides, provide an interesting subject for investigation when more of the specific substances become available.

The work of Morgan & Partridge [1941] revealed new methods of degradation of the parent antigenic complex, which do not involve high temperatures or extremes of acidity. These have been used successfully to prepare undegraded polysaccharides from *S. typhimurium*, which were intermediate in properties and composition between the parent antigenic complex and the degraded polysaccharide. The undegraded polysaccharides possess the remarkable property of forming stable complexes with proteins, from which the latter are not precipitable by acid protein precipitants. The alkali- and phenol-dissociation products (undegraded polysaccharides) display an important difference in that the former is non-antigenic, whilst the latter still stimulates formation of precipitins in rabbits. This antigenicity is thought to be due to residual traces of undecomposed antigenic complex.

Morgan & Partridge also showed how the polysaccharide and conjugated protein components of *Bact. dysenteriae* (Shiga), lacking specific antigenicity, could be recombined to form complexes which gave rise to powerful specific antibodies on injection into animals. This has been confirmed and extended to *S. typhimurium*; mixture of the non-antigenic alkali-dissociated polysaccharide and the conjugated protein (feebly antigenic but with no *S. typhimurium* specificity) in alkaline solution, followed by acidification, produced a powerfully antigenic

complex which gave rise to *S. typhimurium* precipitins and agglutinins on injection into rabbits. Ultracentrifugal studies of mixtures of the undegraded polysaccharide with albumin also clearly showed the presence of a new complex.

SUMMARY

1. By means of ethanol and ammonium sulphate fractionation from aqueous solution the antigenic complex of *Salmonella typhimurium* has been obtained in a form which is believed to represent a chemical entity.

2. On acetic acid hydrolysis the antigenic complex is broken down into four components: (*a*) a specific polysaccharide (69 %), (*b*) an insoluble conjugated protein (16 %), (*c*) a mixed lipid fraction (3–4 %) and (*d*) an alcohol-soluble acetyl-polysaccharide (about 8 %).

3. The specific polysaccharide is non-antigenic. It is dextrorotatory, $[\alpha]_D^{19°}$, $+103° \pm 1°$; it is free from nitrogen and yields 93 % of reducing sugars on acidic hydrolysis. The polysaccharide is free from ketose, pentose and uronic acid residues, and yields 31 % *d*-glucose, 21·5 % *d*-mannose and 19 % *d*-galactose on hydrolysis.

4. The antigenic complex has also been dissociated by means of precipitation from weakly alkaline solution, yielding a small amount of an amphoteric protein, and a non-antigenic 'undegraded polysaccharide' which reacts specifically with *Salmonella typhimurium* antiserum to high dilutions. This polysaccharide has the power of forming complexes with proteins such that the protein becomes no longer precipitable by acidic protein precipitants.

The author wishes to thank Prof. Sir Robert Robinson for his interest in this work and Imperial Chemical (Pharmaceuticals) Limited for permission to publish this account of it.

ADDENDUM

Examination in the Ultracentrifuge

By A. G. OGSTON (with technical assistance by Mrs P. M. Hodson),
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Various samples derived from the antigen of *Salmonella typhimurium* were examined in a Svedberg oil-turbine ultracentrifuge, by the 'diagonal schlieren' method of Philpot [1938]; where it was possible, the total refractive increments of the dissolved substances were measured, and the refractive increments appearing in different boundaries determined by integration of areas in the diagrams [Philpot, 1939].

(1) *'Degraded' polysaccharide:* 1 % solution unbuffered, in 0·1M NaCl; $S^w_{20} = 1{\cdot}25 \times 10^{-13}$ is close to the value given by Philpot [1942] for the polysaccharide derived from *Eberth. typhosa* Ty 2. The form of the sedimentation diagram (Fig. 3) indicates fair homogeneity and probably a not very asymmetric shape of particle (less so than that of *Eberth. typhosa* polysaccharide): if we assume a partial specific volume of 0·62, this gives a minimum molecular weight of 3600. Only 80 % of the total refractive increment appears in the boundary, which shows that there is some non-sedimenting or highly heterogeneous material.

(2) *Undegraded polysaccharide:* 0·5 % in 0·01M phosphate buffer, pH 6·8 and 0·2M NaCl: this presents a very different picture. There are at least three molecular species present of $S^w_{20} = 13{\cdot}1$, 7·6 and $1{\cdot}72 \times 10^{-13}$ respectively: the diagram (Fig. 3) indicates also some rather heterogeneous material of sedimentation constant greater than 13×10^{-13}, and possibly a small component of sedimentation constant less than $1{\cdot}72 \times 10^{-13}$. The form of boundary shown by the component of $S^w_{20} = 7{\cdot}6$ suggests that its particles are asymmetric. The component of $S^w_{20} = 1{\cdot}72$ may be identical with the 'degraded' polysaccharide.

(3) In view of the remarkable power of the undegraded polysaccharide to prevent the precipitation of proteins by trichloroacetic acid, a solution of it with crystalline horse serum albumin was compared with solutions of the polysaccharide and of albumin separately. The solution was 0·5 % with respect to polysaccharide, 1 % with respect to albumin, in 0·01M phosphate buffer pH 6·8 and 0·2M NaCl. The runs were carried out as nearly as possible identically with respect to speed, temperature, etc. and photographs were taken at corresponding times, with identical settings of the optical system (Fig. 4). The mixture contains two main components: one, of $S^w_{20} = 3{\cdot}57 \times 10^{-13}$, comprises 70 % of the total, has a sedimentation constant significantly lower than that of serum albumin alone ($4{\cdot}11 \times 10^{-13}$) and the shape of the boundary suggests a more asymmetric particle; the other, of $S^w_{20} = 5{\cdot}11 \times 10^{-13}$, comprises 30 % of the total and appears to be somewhat heterogeneous. There is no sign in the diagram of the mixture of any of the components of the polysaccharide, and the results provide clear evidence of complex formation be-

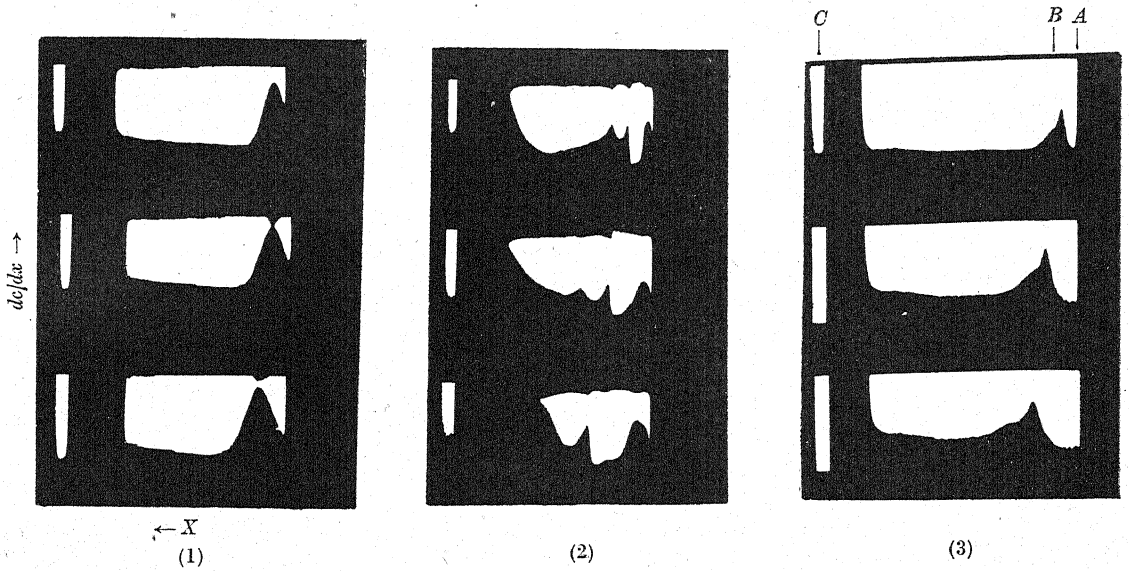

Fig. 3. (1) degraded polysaccharide: 1%, 20 min. intervals, 1080 r.p.s.; (2) undegraded polysaccharide: 0·5%, 10 min. intervals, 1000 r.p.s.; (3) antigen: 0·5%, 10 min. intervals, 370 r.p.s.

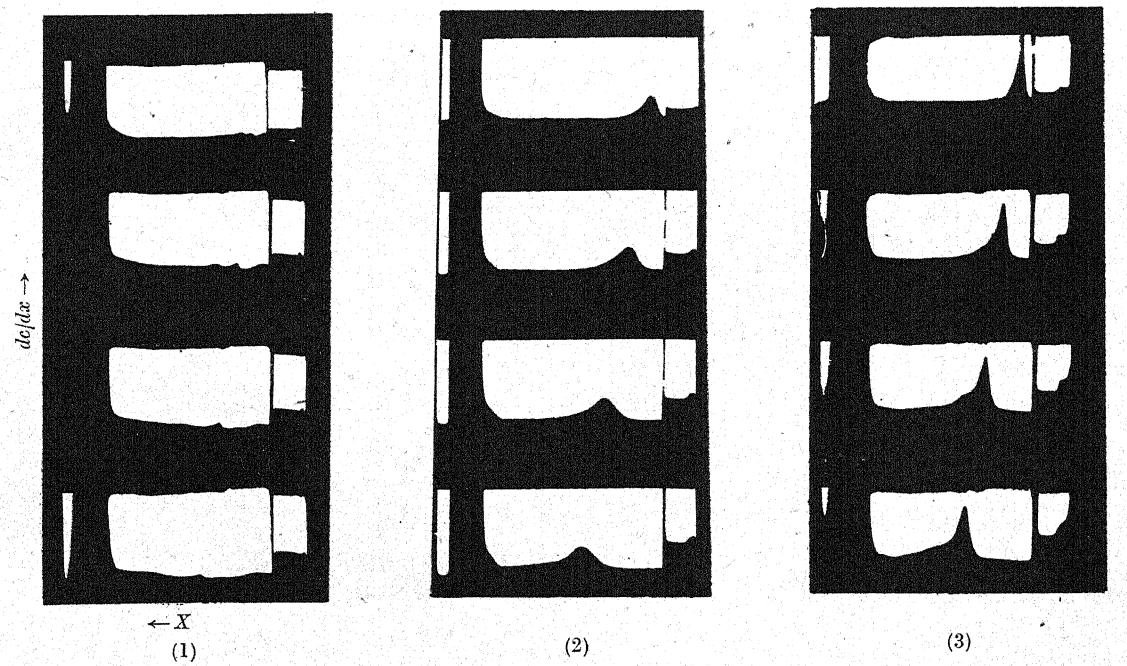

Fig. 4. (1) undegraded polysaccharide: 0·5%; (2) serum albumin: 1%; (3) undegraded polysaccharide: 0·5% + serum albumin, 1%, 20 min. intervals, 1050 r.p.s. Identical times and optical settings for all three sets of diagrams.

X = distance from centre of rotation; dc/dx = concentration gradient; A = meniscus; B = sedimentation boundary; C = index.

tween the protein and all components of the polysaccharide. This is in agreement with the titration data and the prevention of precipitation of the protein by trichloroacetic acid.*

resembles these rather closely, suggesting a similar type of combination with protein. Again, the main component (70 %) seems to be homogeneous, while the other (30 %) is rather heterogeneous. Only

Table 4. *Sedimentation constants of* Salmonella typhimurium *fractions*

Substance	Sedimentation constant ($S^{w}_{20} \times 10^{-13}$)	M (assumed value of V* in brackets)	Fraction of total refracting material	Specific refractive increment
Degraded polysaccharide	1·25	3,600 (0·62)	0·82	0·0012
Undegraded polysaccharide (0·5 %)	1·72	5,800 (0·62)	—	0·0012
	7·60	54,000 (0·62)		
	13·10	117,000 (0·62)		
Serum albumin	4·11	70,000	0·73	—
0·5 % undegraded polysaccharide + 1 % serum albumin	3·57	28,000 (0·71)	0·60	—
	5·11	48,000 (0·71)	0·23	
Antigen (0·526 %)	56·8	$1·5 \times 10^{6}$ (0·68)	0·31	0·00115
	79·7	$2·5 \times 10^{6}$ (0·68)	0·14	

* Partial specific volume.

(4) The bacterial antigen (Fig. 3) shows two sedimentation boundaries of $S^{w}_{20} = 79·7$ and $56·8 \times 10^{-13}$. Although the sedimentation rates are much greater than those of the polysaccharide-serum albumin mixture, the general character of the components

* The horse serum albumin was prepared from horse serum in 1938 by the method of Hewitt [1938] somewhat modified. It was free from carbohydrate and appeared to be homogeneous. It was lyophil dried. In the course of five years it seems to have become heterogeneous to some extent; the specific refractive increment in the boundary is only 0·00136 compared with 0·00185 when it was made. The sedimentation constant of the homogeneous part remains unchanged.

about 50 % of the total refracting material is present in these components. There is also a small amount of material which sediments very slowly.

The results are summarized in Table 4: column 2 gives the sedimentation constant in water at 20°; column 3 gives estimates of the molecular weights: it must be emphasized that these are calculated for spherical particles on the basis of the assumed values of the partial specific volumes given in brackets, and therefore do no more than indicate the orders of magnitude; column 4 gives the fraction of total refracting material appearing in the respective boundaries.

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The Reduction of Methaemoglobin by Ascorbic Acid

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In idiopathic methaemoglobinaemia, of which we have investigated some cases, the occurrence of an atypical form of methaemoglobin abnormally resistant to reduction has been postulated by Lian, Frumusan & Sassier [1939] as an aetiological factor. It thus became of interest to examine the evidence which has been put forward by Vestling [1942] against the unitary view of methaemoglobin. This author found that methaemoglobin made from haemoglobin by the action of nitrite was much less readily reduced by ascorbic acid than methaemoglobin formed by the action of ferricyanide, and suggested that these two spectroscopically similar products [Austin & Drabkin, 1935] differed chemically. The reaction is also of interest because it has been applied clinically by Deeny, Murdock & Rogan [1943] in the treatment of idiopathic methaemoglobinaemia.

The experiments reported here indicate that there is no chemical difference between nitrite- and ferricyanide-prepared methaemoglobin, so far as reduction by ascorbic acid is concerned, and that the differences observed by Vestling are due to the presence of ferrocyanide.

METHODS

Crystalline horse haemoglobin was prepared following Taylor & Hastings [1939], and was preserved in the ice-chest in contact with toluene. Methaemoglobin (MHb) was determined by the method of Evelyn & Malloy [1938], using a Hilger Spekker absorptiometer with filter no. 1 (red), of set H. 455, transmitting light of wave-lengths greater than 620 mμ. A calibration curve was prepared by using known amounts of MHb prepared from haemoglobin solutions which had been standardized with the Van Slyke-Neill manometric apparatus.

Determinations of total pigment were made by the method of Clegg & King [1942], again using the Spekker absorptiometer with filter no. 5 (green), of set H. 455 (maximal transmission between 500 and 580 mμ), and a calibration curve prepared with the aid of the Van Slyke apparatus.

In order to follow the course of the reaction between MHb and ascorbic acid, haemoglobin solutions having a concentration of about 10 g./100 ml. were prepared with M/20 phosphate buffer, pH 7·4, and partially converted to MHb by the addition of either one equivalent of $NaNO_2$ or of $K_3Fe(CN)_6$. The resulting solution (2 ml.) was placed in the main chambers of a series of Barcroft manometric flasks of the usual type. The required amount of *l*-ascorbic acid (B.D.H. synthetic), dissolved in M/3 phosphate buffer, pH 7·4 was contained in Keilin tubes. The manometers were filled with a mixture of 20% CO (prepared from formic acid), and 80% N_2. After equilibration by 15 min. shaking in the water-bath, the ascorbic acid was added from the Keilin tubes and the reaction followed by the uptake of CO by the haemoglobin formed from MHb. At the end of an experiment, the final concentration of MHb in each flask was estimated with the absorptiometer.

A few experiments were carried out in the presence of N_2 only; here a modified Thunberg tube was used with a tap attached to the bottom, by means of which samples of solution could be withdrawn during the course of an experiment. The MHb solution was placed in the main tube, the ascorbic acid in the side arm, and the vessel repeatedly evacuated and refilled with N_2. The solutions were then mixed and portions removed at intervals for determination of MHb.

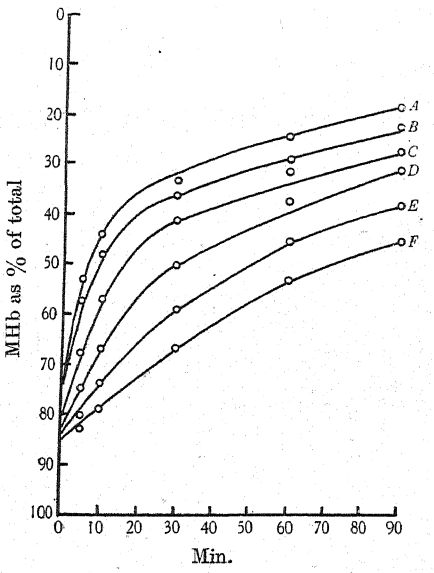

Fig. 1. The effect of ferrocyanide on the reduction of nitrite-methaemoglobin by ascorbic acid. In all manometers, 6·6 μmol. methaemoglobin and 6·6 μmol. ascorbic acid. $K_4Fe(CN)_6$: *A*, 13·2 μmol.; *B*, 6·6 μmol.; *C*, 3·3 μmol.; *D*, 1·7 μmol.; *E*, 0·8 μmol.; *F*, nil. Temp. = 28°. Total vol. in each, 2·2 ml.

RESULTS

The effect of ferrocyanide on the rate of reduction of nitrite-methaemoglobin. The effect of the addition of varying amounts of $K_4Fe(CN)_6$ on the reaction between nitrite-methaemoglobin (nitrite-MHb) and ascorbic acid is shown in Fig. 1. With the amount

of CO absorbed in the first 5 min. as a measure of the velocity of the reaction, it was found that the acceleration produced was directly proportional to the amount of ferrocyanide added until the level of one equivalent was reached; beyond this point the relation does not hold, in part because a 5 min. period is too long to give a true measure of the initial velocity of the reaction when this is high. It will be noticed that in Fig. 1 the amounts of MHb present initially are different. When ferrocyanide acts upon a solution containing MHb, the formation of haemoglobin and ferricyanide takes place until equilibrium is reached. Since the ferrocyanide was added to the MHb solutions before the beginning of the experiment, this reaction was largely completed during the equilibration period and led to a diminished initial MHb concentration in the manometers containing large amounts of ferrocyanide.

Comparison between ferricyanide-methaemoglobin and nitrite-methaemoglobin. The effect of the addition of ferrocyanide on the rate of reduction of nitrite-MHb made it likely that the observed difference between it and ferricyanide-methaemoglobin (ferricyanide-MHb) might be due to the presence of ferrocyanide formed during the preparation of the latter. To test this point, a concentrated solution of ferricyanide-MHb was dialyzed in a collodion sac against tap water for 24 hr. The resulting solution had a MHb concentration of 5·2 g./100 ml.; the rate of reduction by two equivalents of ascorbic acid with and without one equivalent of potassium ferrocyanide was compared with the rate of reduction of a nitrite-MHb solution of equal concentration. The results are shown in Fig. 2 and support the view that there is no essential difference between the two types of MHb.

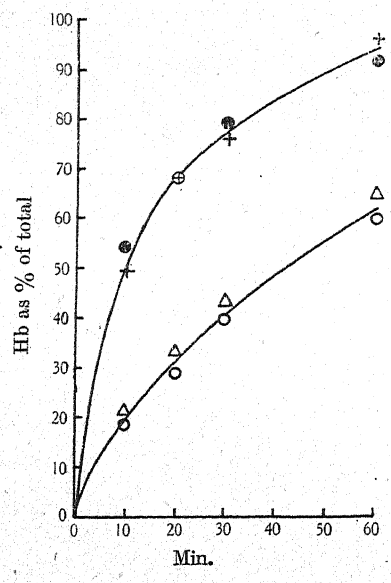

Fig. 2. Comparison between nitrite-methaemoglobin and ferricyanide-methaemoglobin. In each manometer 6·2 μmol. methaemoglobin and 6·2 μmol. ascorbic acid. Total volume 2·2 ml. Temp. = 37°. o Dialyzed ferricyanide-MHb. △ Nitrite-MHb. + Dialyzed ferricyanide-MHb + 6·2 μmol. $K_4Fe(CN)_6$. • Nitrite-MHb + 6·2 μmol. $K_4Fe(CN)_6$.

The mechanism of the catalytic action of ferrocyanide on the reduction of methaemoglobin. The most obvious suggestion is that the iron in the ferrocyanide acts as a catalyst by being alternately oxidized and reduced according to the following reactions:

$$Fe^{+++}\,(MHb) + \text{ferrocyanide} \rightarrow Fe^{++}\,(Hb) + \text{ferricyanide}.$$

$$\text{Ferricyanide} + \text{ascorbic acid} \rightarrow \text{ferrocyanide} + \text{dehydroascorbic acid}.$$

In order that the first reaction may proceed from left to right, it is a necessary condition that the ratio ferri-/ferro-cyanide be such that the e.m.f. of the system ($E_0' = +406$ mV. [Michaelis, 1933]), shall be less positive than that of the system MHb/Hb ($E_0' = +140$ mV. at pH 7 [Taylor & Hastings, 1939]). If equimolecular amounts of MHb and Hb were present, fulfilment of this condition would require that the ratio ferri-/ferro-cyanide should be less than 1:27,000. This consideration, however, does not rule out the possibility of this reaction in the experiments just quoted, because these were carried out in the presence of CO, which, by combining with free Hb, so alters the value of the quotient MHb/Hb that the effective E_0' of the system, as calculated from the equilibrium reached in the presence of ferrocyanide, becomes about +380 mV. It would be expected that in the absence of a gas with which haemoglobin combines, the catalytic effect of ferrocyanide would be much reduced or would disappear. Repetition of the experiments shown in Fig. 2 in an atmosphere of N_2 led to results which were, within experimental error, precisely similar to those obtained in the presence of CO, suggesting, therefore, that ferrocyanide, as such, does not act by being alternately oxidized and reduced. The true nature of the catalysis remains obscure.

Catalysis of the reduction of methaemoglobin by metals. The existence of ferrocyanide catalysis suggested that other substances might share this property and it has, in fact, been found that copper and iron both exert a considerable effect on the rate of reduction. The effect of $FeSO_4.(NH_4)_2SO_4.6H_2O$ and $CuSO_4$ is shown in Table 1. Some part of the 'normal' reaction between MHb and ascorbic acid is probably due to traces of metals present in the solutions, for it was found that saturation with 8-hydroxyquinoline (combining with copper) and $\alpha\alpha'$-dipyridyl (combining with ferrous iron) reduced the rate of reduction by some 50 and 20 % respectively.

Table 1. *Catalysis of the reduction of methaemoglobin by* Fe *and* Cu

	Metal catalyst (μg./ml.)	Methaemoglobin reduced in 30 min. (μmol.)
	None	3·4
Fe	25	5·1
	50	5·4
Cu	25	4·7
	50	5·1

Methaemoglobin (12·0 μmol. in 2 ml.) + ascorbic acid (two equiv.). Vol. = 2·2 ml.; temp. = 28°. The data given are the means of duplicate determinations.

The mechanism of the reaction between nitrite-methaemoglobin and ascorbic acid. The reaction between MHb and ascorbic acid appears to be satisfactorily described by the equation for a bimolecular reaction, as shown by the reasonably constant values of k (velocity constant), obtained by substitution in the bimolecular formula. Table 2

Table 2. *Velocity constant for the reduction of methaemoglobin by ascorbic acid*

Time (hr.)	CO uptake (μl.)	k
0·17	14	1·43
0·50	34	1·33
1·0	50	1·15
1·5	63	1·13
2·0	73	1·15

Methaemoglobin (6·0 μmol. in 2 ml.) + ascorbic acid (one equiv.). Vol. = 2·2 ml.; temp. = 37°.

shows the analysis of one experiment. Determination of the order of the reaction by observation of the time required to reach 50 % reduction, starting with equivalent quantities of reactants at varying concentrations, also supported this conclusion (Table 3).

Table 3. *Inverse proportionality between concentration of reactants and time required to reach* 50 % *reduction of methaemoglobin*

Initial [MHb] (g./100 ml.)	Time of 50 % reduction (t, min.) Individual results	Mean	Product: [MHb] × t
10·0	50, 50	53	530
8·4	67, 66, 60	63	528
5·0	116, 102	109	545
4·2	116, 112, 108	112	470
		Av.	518

The effect of ascorbic acid concentration on the rate of reduction of methaemoglobin. The initial rate of reaction with constant MHb concentration should be proportional to the ascorbic acid concentration. An attempt to verify this relation was made by measuring the gas uptake during the first 10 min. of the reaction, with varied amounts of ascorbic acid in the presence of two equivalents of glutathione, which, while itself reacting only slowly with MHb, maintained the ascorbic acid in the reduced form and enabled measurable gas uptakes to be obtained with the smaller concentrations of ascorbic acid. The results are shown in Table 4. The apparent

Table 4. *Effects of ascorbic acid concentration and 8-hydroxyquinoline on reduction of methaemoglobin*

Ascorbic acid (μmol.)	μl. CO absorbed in 10 min. 8-Hydroxyquinoline absent: Gross	Net	8-Hydroxyquinoline present: Gross	Net	Inhibition (%)
—	11	—	10	—	—
0·8	31	20	16	6	70
1·5	43	32	20	10	69
3	54	43	25	15	65
6	73	62	36	26	58
12	89	78	45	35	55

Methaemoglobin (12 μmol.) + glutathione (24 μmol.). Vol. = 2·2 ml.; temp. = 37°.

velocity with small concentrations of ascorbic acid was much in excess of expectation, while the inhibition produced by saturation with 8-hydroxyquinoline was more marked in the case of low concentrations of ascorbic acid, suggesting that the reaction usually observed is made up of two components, the one a direct reaction between ascorbic acid and MHb, the other a reaction catalyzed by traces of metals in solution, the latter being little affected by increase of the ascorbic acid concentration beyond a low limiting value.

DISCUSSION

While similar behaviour towards ascorbic acid does not necessarily indicate that nitrite-MHb and ferricyanide-MHb are identical, the present results remove the experimental basis for the contrary view.

It may be remarked that Vestling [1942] found an equilibrium was reached between ferricyanide-MHb and two equivalents of ascorbic acid when 83–95 % of the MHb had been reduced. In this series of experiments, it has been found that when sufficient time is allowed (24 hr. at room temperature) reduction proceeds so far that the residual MHb does not amount to more than 1–2 % of the total pigment present. In view of the large difference between the potentials of the ascorbic acid and MHb/Hb systems in the presence of a gas combining with Hb, and the instability of dehydroascorbic acid, it would appear that the concept of an equilibrium must be largely illusory, and that Vestling's results may best be explained by the

occurrence of secondary oxidative changes (for his experiments were not carried out under anaerobic conditions), and by the probability that insufficient time was allowed for the reaction to go nearly to completion.

Extrapolation from the results obtained *in vitro* suggests that the rate of reduction of methaemoglobin, which could be expected with the concentrations of ascorbic acid likely to occur *in vivo*, would be small as compared with the rate of reduction of the pigment brought about by the enzymic mechanism of normal erythrocytes [Cox & Wendel, 1942]. The clinical use of ascorbic acid in the treatment of methaemoglobinaemia would thus appear to be limited to cases in which the normal enzymic mechanism for the removal of methaemoglobin is absent or has become suppressed.

SUMMARY

1. It has been confirmed that ferricyanide-methaemoglobin, as ordinarily prepared, is reduced more rapidly by ascorbic acid than is nitrite-methaemoglobin.

2. This difference is due to the catalytic effect of ferrocyanide on the reduction of methaemoglobin. There is thus no basis for the supposition that this effect indicates a chemical difference between the two forms of methaemoglobin.

3. Iron and copper salts produce similar catalytic effects.

4. The reaction between ascorbic acid and methaemoglobin is described by the equation for a bimolecular reaction.

The author wishes to thank Prof. D. C. Harrison for much helpful criticism and advice.

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The Assimilation of Glucose and Galactose in the Liver

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In this paper an attempt is made to examine the intermediary metabolism of glucose and galactose in the liver of the intact animal. *In vitro* experiments [Cori, Cori & Schmidt, 1939; Ostern, Herbert & Holmes, 1939] have proved that glucose-1-phosphate and hexose-6-phosphate are intermediaries in the glucose and glycogen metabolism of the liver. Both galactose-1-phosphate and glucose-1-phosphate have been found in the livers of rabbits assimilating galactose [Kosterlitz, 1937; 1943*a*]. In the present investigation, in addition to the phosphoric esters, glucose, galactose, and glycogen were estimated in the livers of rats at varying intervals after ingestion of glucose or galactose.

METHODS

General. Male rats from the Rowett Research Institute were kept on the stock diet. Prior to the experiment they were fasted for 24 hr., and then fed by stomach tube with either 1·5 ml. of 60% (w/v) galactose solution/100 g. body weight, or with 1·5 ml. of 55% (w/v) glucose solution. After 30, 60, 90 or 120 min. the rats were killed by stunning and the livers excised. After about 0·5 g. liver had been placed in 30% KOH for glycogen estimation, the remainder was frozen in liquid O_2; samples of the frozen liver were used for the various estimations.

Estimation of glucose and galactose. The $HgSO_4$-$BaCO_3$ method of West, Scharles & Peterson [1929] was used for the preparation of protein-free filtrates. Absence of traces of Ba^{++} and Hg^{++} was ensured by the addition of two drops of saturated solution of K_2SO_4 and of a small quantity of Zn powder. Glucose and galactose were estimated in the filtrate by the fermentation method previously described [Kosterlitz, 1937].

Estimation of glycogen. Our micro-adaptation of Pflüger's method was used [Kosterlitz, 1933], with 30% KOH as digestion fluid.

Estimation of reducing phosphoric esters, glucose-1-phosphate and galactose-1-phosphate. This method has previously been described [Kosterlitz & Ritchie, 1943]. The results are given under the following headings: (1) 'Initial reduction',

which is an approximate measure of the reducing phosphoric esters present. Since the nitrogenous esters have been removed, this fraction is composed mainly of equilibrium ester (glucose-6-phosphate and fructose-6-phosphate), possibly together with small quantities of pentose-phosphates. If galactose-6-phosphate should be formed, it also would contribute to the 'initial reduction'; but so far there is no evidence that this ester is formed in the liver during galactose assimilation. (2) Increase in reducing power, calculated as glucose, after 3 min. hydrolysis at 100° ($Red._{100°}$) and after 30 min. hydrolysis at 50° ($Red._{50°}$), and increase in inorganic P (uncorrected) after 3 min. hydrolysis at 100° ($P_{100°}$) and 30 min. hydrolysis at 50° ($P_{50°}$). The values thus obtained are used for characterizing the 1-esters present: glucose-1-phosphate has $Red._{50°}/Red._{100°} = P_{50°}/P_{100°} = 0{\cdot}286$ and $Red._{100°}/P_{100°} = Red._{50°}/P_{50°} = 5{\cdot}81$, while galactose-1-phosphate has $Red._{50°}/Red._{100°} = 0{\cdot}775$, $P_{50°}/P_{100°} = 0{\cdot}765$, $Red._{100°}/P_{100°} = 4{\cdot}63$, and $Red._{50°}/P_{50°} = 4{\cdot}70$.

In experiments on the addition of 1-esters to CCl_3COOH extracts of liver only about 80% of the added material was recovered: in this paper no correction has been made for this loss of 20%.

Significance of results. The statistical methods described by Fisher [1941] were used.

found for $Red._{50°}/Red._{100°}$, $Red._{100°}/P_{100°}$, and particularly $Red._{50°}/P_{50°}$ suggested, however, that besides glucose-1-phosphate an unidentified substance was present, which was probably not a phosphoric ester. It was about as sensitive to acid hydrolysis as galactose-1-phosphate and there was no further increase in reducing power when the hydrolysis at 100° was extended from 3 to 20 min.

Some variation was found in the glycogen contents of the livers. There was, however, a remarkably close relationship between the glycogen and glucose contents, which varied in the same direction and were sometimes almost identical. This phenomenon was absent in rats fed either glucose or galactose. Similarly, Guest [1941] found in fasted but not in fed rats that the mean blood sugar concentrations were directly proportional to the logarithms of the liver glycogen concentrations.

Rats fed with glucose (Tables 1 and 2). The glycogen content of the liver increased with prolongation of the period of glucose absorption, but otherwise

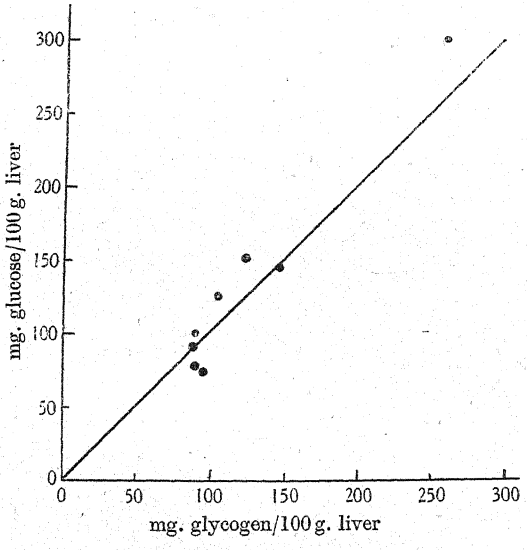

Fig. 1.

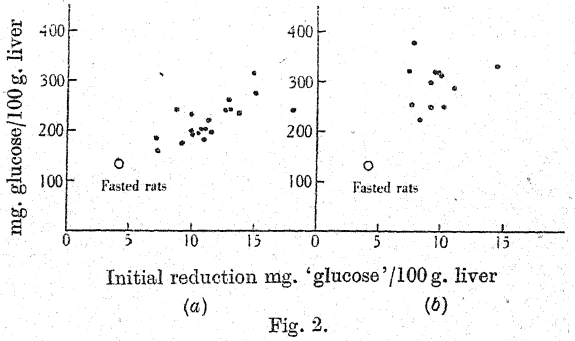

Fig. 2.

Fig. 1. Relationship between glucose and glycogen contents of livers of rats fasted for 24 hr.

Fig. 2. Correlation between glucose and reducing phosphoric ester levels in the livers of rats fed with (*a*) galactose, and (*b*) glucose. Correlation coefficients: (*a*) $r = +0{\cdot}6926$, $P < 0{\cdot}01$; (*b*) $r = +0{\cdot}1457$, $P > 0{\cdot}1$.

RESULTS

Rats fasted for 24 hr. (Fig. 1 and Table 2). Very small quantities of acid-labile phosphoric esters appear to be present in the liver. The ratio $P_{50°}/P_{100°}$ was 0·225, agreeing approximately with the value calculated for glucose-1-phosphate. The high values

the duration of the absorption had no influence on the results. For the purpose of comparing the reducing phosphoric ester and glucose-1-phosphate levels of the glucose-fed rats with those of the fasted ones, the results obtained after feeding glucose were pooled. There was a highly significant increase in both esters after glucose administration. On the

Table 1. *Data relating to carbohydrate and phosphoric ester contents of livers of rats given different sugars*

Sugar fed.	Time elapsing between feeding of sugar and death of animal (min.)	No. of rats in group	Mean weight of group (g.)	Liver: Weight (g./100 g. body wt.)	Liver: Glycogen content (g./100 g.)	Liver: Glucose content (g./100 g.)	Liver: Galactose content (g./100 g.)	Liver: Initial reducing power (approx. measure of reducing, i.e. hexose-6-phosphoric esters) (mg. 'glucose'/100 g.)	Liver: Increase in reducing power or inorganic P content (approx. measure of hexose-1-phosphates) (mg./100 g.): After 3 min. hydrolysis at 100°: 'Glucose'	After 3 min. hydrolysis at 100°: Inorganic P	After 30 min. hydrolysis at 50°: 'Glucose'	After 30 min. hydrolysis at 50°: Inorganic P
Glucose	30	3	293 ±16	2·86 ±0·03	0·331 ±0·006	0·331 ±0·023	—	8·0 ±0·52	2·25 ±0·22	0·17 ±0·051	1·25 ±0·13	0·07 ±0·039
	60	3	278 ±21	2·85 ±0·05	0·741 ±0·102	0·284 ±0·017	—	9·5 ±1·02	2·7 ±0·09	0·185 ±0·044	1·4 ±0·14	0·045 ±0·035
	90	3	292 ±34	2·90 ±0·15	1·52 ±0·075	0·277 ±0·027	—	11·2 ±1·63	2·3 ±0·15	0·22 ±0·032	0·9 ±0·09	0·065 ±0·035
	120	3	280 ±25	3·11 ±0·09	2·06 ±0·173	0·285 ±0·031	—	9·15 ±0·48	2·65 ±0·12	0·245 ±0·018	1·5 ±0·24	0·065 ±0·023
Galactose	30	5	319 ± 9	2·75 ±0·06	0·259 ±0·047	0·218 ±0·028	0·181 ±0·013	10·5 ±1·61	4·35 ±0·26	0·56 ±0·036	2·7 ±0·28	0·315 ±0·010
	60	5	296 ±12	2·59 ±0·09	0·469 ±0·089	0·214 ±0·016	0·227 ±0·020	11·4 ±0·60	4·3 ±0·33	0·60 ±0·033	2·9 ±0·24	0·36 ±0·002
	90	5	301 ±19	2·70 ±0·07	0·719 ±0·112	0·239 ±0·011	0·281 ±0·026	11·5 ±1·13	5·0 ±0·15	0·57 ±0·048	3·05 ±0·17	0·34 ±0·020
	120	5	287 ±12	2·86 ±0·14	0·825 ±0·066	0·206 ±0·010	0·277 ±0·025	12·2 ±1·51	4·85 ±0·38	0·71 ±0·038	3·15 ±0·34	0·455 ±0·046

In the expression 293 ±16, 16 is the standard deviation of the mean.

Table 2. *Interpretation of carbohydrate and phosphoric ester analyses*

	Liver: Glucose (g./100 g.)	Liver: Hexose-6-phosphates. Initial reducing power (mg. 'glucose'/100 g.)	Liver: Hexose-1-phosphates: Increase in reducing power or inorganic P content (mg./100 g.): After 3 min. hydrolysis at 100°: 'Glucose' (=$Red._{100°}$)	After 3 min. hydrolysis at 100°: Inorganic P (=$P_{100°}$)	After 30 min. hydrolysis at 50°: 'Glucose' (=$Red._{50°}$)	After 30 min. hydrolysis at 50°: Inorganic P (=$P_{50°}$)	$Red._{50°}/Red._{100°}$	$P_{50°}/P_{100°}$	$Red._{100°}/P_{100°}$	$Red._{50°}/P_{50°}$
Mean of 10 fasted rats	0·133 ±0·026	4·13 ±0·40	1·54 ±0·09	0·089 ±0·009	0·84 ±0·09	0·020 ±0·007	0·545	0·225	17·3	42·0
Mean of 12 glucose-fed rats	0·294 ±0·012	9·45 ±0·56	2·48 ±0·09	0·205 ±0·019	1·28 ±0·10	0·060 ±0·015	0·516	0·293	12·1	21·3
Mean of 20 galactose-fed rats	0·219 ±0·009	11·4 ±0·60	4·63 ±0·15	0·610 ±0·023	2·94 ±0·13	0·367 ±0·017	0·635	0·602	7·6	8·0
Mean of glucose-fed minus mean of fasted rats	0·161	5·32	0·94	0·116	0·44	0·040*	0·468	0·345	8·1	11·0
Mean of galactose-fed minus mean of fasted rats	0·086	7·27	3·09	0·521	2·10	0·347	0·680	0·666	5·93	6·05
Mean of galactose-fed minus mean of glucose-fed rats	−0·075	1·95*	2·15	0·405	1·66	0·307	0·772	0·758	5·31	5·41
Calculated for: Glucose-1-phosphate							0·286	0·286	5·81	5·81
Galactose-1-phosphate							0·775	0·765	4·63	4·70

In the expression 0·133 ±0·026, 0·026 is the standard deviation of the mean.

* The differences of the means are all significant, the values marked by an asterisk having P between 0·05 and 0·02 and the others $P < 0·01$ [Fisher, 1941].

other hand, no correlation was found between liver glucose, glycogen, reducing phosphoric esters and glucose-1-phosphate. The ratios Red.$_{50°}$/Red.$_{100°}$ and Red./P were somewhat smaller than those in the fasted rats but still greater than the values calculated for glucose-1-phosphate. Similarly the values obtained from the differences of the means of the glucose-fed and fasted rats were still too high for glucose-1-phosphate. This may indicate an increase in the unknown acid-labile substance already found in the livers of fasted animals, but the number of observations is too small to make any definite statement on this point.

Rats fed with galactose (Tables 1 and 2; Fig. 2). There was a gradual increase in the liver galactose and glycogen with increased duration of galactose absorption, which had no other significant influence on the results, with the possible exception of the 120 min. P values. The glucose levels were lower than those in the rats fed glucose. The reducing phosphoric esters (pooled results on 20 rats) were slightly higher in the animals fed galactose than in those fed glucose. There was a highly significant positive correlation between glucose and reducing phosphoric esters which was absent in the glucose-fed rats (Fig. 2). The 1-phosphates were considerably higher than in the fasted and the glucose-fed animals. Both ratios, Red.$_{50°}$/Red.$_{100°}$ and $P_{50°}/P_{100°}$, were near 0·6, a value indicating a mixture of approximately 1 part glucose-1-phosphate (0·203 mg. P/100 g. liver) and 2 parts galactose-1-phosphate (0·406 mg. P/100 g. liver) [Kosterlitz & Ritchie, 1943]. The ratios Red./P were too high for either glucose-1-phosphate or galactose-1-phosphate. This possibly indicates an increase in the unknown acid-labile substance, already noted in the case of the glucose-fed animals. If the ratios were calculated from values obtained by deducting the means of the values for the fasted rats from those for the galactose-fed ones, a small increase of the ratios Red.$_{50°}$/Red.$_{100°}$ and $P_{50°}/P_{100°}$ was found, and a decrease of the Red./P ratios. The data thus obtained indicated a mixture of approximately 1 part glucose-1-phosphate (0·104 mg. P/100 g. liver) and 4 parts galactose-1-phosphate (0·416 mg. P/100 g. liver). From these calculations, and the values given for the glucose-fed rats (Table 2), one may conclude that in the galactose-fed rats 0·41 mg. galactose-1-phosphate P and 0·2 mg. glucose-1-phosphate P were present in 100 g. liver, and that in both glucose-fed and galactose-fed rats the same quantity of glucose-1-phosphate was formed, namely, that corresponding to 0·11 mg. P. This conclusion is further supported by the fact that the ratios calculated from the differences between the mean values for the galactose-fed and those for the glucose-fed rats were very close to the ratios calculated for galactose-1-phosphate: Red.$_{50°}$/Red.$_{100°}$ was 0·772 and $P_{50°}/P_{100°}$ 0·758 instead of 0·775 and 0·765 respectively, and Red.$_{100°}$/$P_{100°}$ 5·31 and Red.$_{50°}$/$P_{50°}$ 5·41 instead of 4·63 and 4·7 respectively; the P content was 0·405 mg./100 g. liver.

DISCUSSION

Rats assimilating glucose. Besides a deposition of glycogen and an increase in glucose, the administration of glucose caused an increase in the reducing phosphoric ester and glucose-1-phosphate levels of the liver. This is in agreement with the generally accepted theory that glycogen formation from glucose is preceded by the formation of glucose-6-phosphate and glucose-1-phosphate [Colowick & Sutherland, 1942]. Apart from glycogen, no metabolite accumulated in the liver during the assimilation of glucose.

Rats assimilating galactose. The deposition of glycogen was much smaller in the galactose-fed than in the glucose-fed rats, a fact already frequently observed [for literature see Deuel, 1936].

The glucose content of the liver was higher than in the fasted rats but lower than in the glucose-fed ones. The rise in liver glucose after galactose administration demonstrates the conversion of galactose to glucose. Harding, Grant & Glaister [1934] could find no significant increase in liver glucose after the administration of galactose, but the quantities given were much smaller than those used here.

Although the experimental evidence available is incomplete, it is improbable that the reducing phosphoric ester fraction isolated from the liver during galactose assimilation contains galactose-6-phosphate. This fraction has not yet been specially examined for the presence or absence of galactose-6-phosphate; however, prolonged acid hydrolysis did not liberate more galactose from the hexosemonophosphate fraction than a brief period of hydrolysis which is sufficient to hydrolyze galactose-1-phosphate but which hardly affects the difficultly hydrolyzable galactose-6-phosphate [Kosterlitz, 1937]. Further, Grant [1935] could not find any evidence for galactose-6-phosphate as an intermediary in the fermentation of galactose by galactose-adapted yeast.

The positive correlation between liver glucose and reducing phosphoric esters in the galactose-fed rats is remarkable, particularly as no such correlation was found in the glucose-fed animals. In the conversion of galactose to glucose, the reducing phosphoric esters may play an important role, glucose-6-phosphate being the most probable precursor of glucose.

Both galactose-1-phosphate and glucose-1-phosphate were found in the livers of rats assimilating galactose; this confirms previous findings on the rabbit [Kosterlitz, 1937; 1943*a*]. Similar quantities

of glucose-1-phosphate were formed during galactose and glucose assimilation, corresponding to 0·11 mg. P or 0·6 mg. glucose/100 g. of liver. If the values for glucose-1-phosphate found during the assimilation of galactose by the rabbit [Kosterlitz, 1943*a*] are expressed in similar terms, a value of 0·16 mg. P/100 g. of liver is obtained. The galactose-1-phosphate content of the rat livers corresponded to 0·41 mg. P or 2·4 mg. galactose/100 g. and that of the rabbit livers to 0·9–2 mg. P or 5–12 mg. galactose/100 g. No explanation can be offered for the difference in reaction of rabbit and rat; the quantity of galactose-1-phosphate found in the liver is possibly related to the galactose tolerance of the species, which is very low in the rat. Kjerulf-Jensen [1942] determined galactose-1-phosphate in the livers of rats assimilating galactose; his values were much higher than those given in the present paper. His method of estimation, however, was not specific for hexose-1-phosphates; the acid-labile P was determined in the fraction of water-soluble Ba-salts isolated from the liver, without removal of the interfering acid-labile P compounds precipitable by Hg acetate [Kosterlitz & Ritchie, 1943]; there was no determination of the simultaneous increase in reducing power, and no estimation of the rate of acid hydrolysis.

No progressive accumulation of galactose-1-phosphate was found in the liver during galactose assimilation, a fact which makes it improbable that the rate of conversion of galactose-1-phosphate to a yet unknown metabolite is slower than that of galactose phosphorylation. Galactose-1-phosphate is very much more resistant to the enzymes present in liver slices, minced liver or aqueous liver extracts than glucose-1-phosphate [Kosterlitz & Ritchie, 1941]. Although the presence of galactose-1-phosphate, the increases in glucose-1-phosphate and reducing phosphoric ester levels, and particularly the correlation found to exist between reducing phosphoric esters and glucose after administration of galactose, suggest that all these substances take part in the conversion of galactose to glucose in the liver, the connecting link between the galactose and glucose compounds is still missing. In an attempt to formulate a tentative hypothesis of galactose fermentation [Kosterlitz, 1943*b*], galactose-1-phosphate was assumed to be converted to hexose-6-phosphate (Robison ester), probably by way of glucose-1-phosphate. This hypothesis was mainly based on the finding that galactose-1-phosphate and glucose-1-phosphate were fermented at almost identical rates by galactose-adapted yeast. Possibly a similar process takes place in the liver during galactose assimilation, the glucose phosphates then forming glycogen and glucose.

SUMMARY

1. The livers of fasting rats, and of those fed with glucose or with galactose, were analyzed for glucose, galactose, glycogen, glucose-1-phosphate, galactose-1-phosphate and reducing phosphoric ester (mainly glucose-6-phosphate and fructose-6-phosphate).

2. In the livers of fasting rats, reducing phosphoric esters and a small quantity of glucose-1-phosphate (0·09 mg. P/100 g.) were present. Further, there was evidence of the presence of a compound which was probably not a phosphoric ester and which yielded reducing substances on acid hydrolysis. In fasted rats, but not in fed ones, there was a very close direct relationship between the glycogen and glucose contents of the livers.

3. In the livers of rats fed with glucose there was an increase in the reducing phosphoric ester and glucose-1-phosphate (+0·11 mg. P/100 g.) levels.

4. During the assimilation of galactose the livers contained galactose-1-phosphate (0·41 mg. P/100 g.). Glucose-1-phosphate was present in quantities similar to those found after glucose administration, the ratio, glucose-1-phosphate formed to galactose-1-phosphate, being approximately 1/3·5. The livers contained less glucose and slightly more reducing phosphoric esters than those of glucose-fed rats. There was a high correlation between the glucose and reducing phosphoric ester levels, which was absent in glucose-fed animals. The significance of these findings for the mechanism of the conversion of galactose to glucose is discussed.

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The Assessment of Vitamin C Nutrition in Man

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As our knowledge of the part played by ascorbic acid in the maintenance of the intercellular architecture grows [Aschoff & Koch, 1919; Dalldorf, 1938] and in turn the importance of the latter in wound healing [Lund & Crandon, 1941; Bartlett, Jones & Ryan, 1942], the need for a reliable and simple assessment of the nutritional status with respect to this vitamin is obvious. A diminished supply of vitamin C-rich foodstuffs in the war-time diet of some adolescents and adults increases the urgency.

Two methods have been widely used: (1) the urinary saturation test, and (2) an examination of the concentration of ascorbic acid in whole blood, white cell-platelet layer or plasma.

Many of the conflicting reports in the literature concerning the urinary saturation test are undoubtedly attributable to the administration of widely different dosages of ascorbic acid ranging from 50 to 1000 mg./day, orally or intravenously in whole or divided doses and to differences in times of collection of urine. Data for normal daily urinary excretion of ascorbic acid vary from zero [Roe & Hall, 1939] to 40 mg. [Portnoy & Wilkinson, 1938].

Whole-blood determinations present some technical difficulties which are not easily offset. Haemolysis causes oxidation of ascorbic acid and inhibition of this by saturation with CO leads to indefiniteness in the end-point of the titration. Determination of the ascorbic acid content of the white cell-platelet layer involves a more complex technique and requires special apparatus which most laboratories would not possess.

It would be of great advantage to replace the somewhat laborious and unsatisfactory technique of the urinary saturation test, even in its most abbreviated form [Harris & Abbasy, 1937], by one determination. We have therefore endeavoured to establish whether a single determination of the plasma ascorbic acid concentration could be used to assay reliably the status of vitamin C nutrition of a subject. Opinion as to the value of isolated determinations of the plasma ascorbic acid content is equally divided; Portnoy & Wilkinson [1938], Ralli & Sherry [1941], and others hold the view that it is of value. The first named say that it may prove to be better than 'saturation' tests, whilst Kellie & Zilva [1939], Storvick & Hauck [1942], among others, hold the opposite view. Our results indicate that the plasma ascorbic acid determination can be used to assay the standard of vitamin C nutrition, in contrast to the unreliability of observations of the so-called 'resting' urinary daily output.

The work here reported on is compiled from data obtained from 50 subjects and falls into two categories: (*a*) the correspondence between the results of urinary 'saturation' tests and those of the determination of plasma ascorbic acid content, and (*b*) the assessment of the nutritional status of the subject with respect to ascorbic acid.

METHODS

Plasma ascorbic acid. 10 ml. of blood were run into a tube containing 0·1 ml. 20% potassium oxalate and 0·05 ml. 5% KCN, and the whole was mixed and immediately centrifuged. To 4 ml. of the plasma, which should show no haemolysis, were added 16 ml. of freshly prepared 3% HPO_3 in glass-distilled water. After centrifugation, 5 ml. portions were titrated with 0·01% 2:6-dichlorophenolindophenol. No difficulty was experienced in determining a stable end-point if the titration was carried out rapidly, and the results were highly reproducible by both of us. Visual titration was found to be as accurate as photoelectric methods [cf. Mindlin & Butler, 1937–8].

Pijoan & Klemperer [1937] added KCN (5 mg.) to inhibit oxidation of ascorbic acid in freshly drawn venous blood. Barron, Barron & Klemperer [1936] considered that addition of KCN may enhance the ascorbic acid value. Golden & Garfinkel [1942], who added ascorbic acid to the plasma, confirmed Borsook, Davenport, Jeffreys & Warner [1936–7] and Kassan & Roe [1940] in finding that ascorbic acid was preserved best in whole blood, especially at low temperatures of the order of 5–8°, whereas once the plasma had been separated little loss occurred in the first 3 hr. at these temperatures, though there was a rapid loss at room temperature. Our experiments on blood containing a high, but physiological, concentration of ascorbic acid, indicate that KCN does not enhance the reduction titre under the conditions employed, but stabilizes the plasma value.

Urine ascorbic acid and dehydroascorbic acid. Urine was collected in dark-coloured Winchesters containing 70 g. HPO_3. 0·05 ml. 0·10% 2:6-dichlorophenolindophenol was titrated with the urine, the latter diluted with a suitable volume of 3% HPO_3 if necessary [cf. Harris & Ray, 1935].

For dehydroascorbic acid a convenient volume of urine was treated with H_2S for 30 min. and then with N_2 for 15 min. after all traces of H_2S had been removed as shown by lead acetate paper. The resulting solution was used to titrate the dye.

The dye solutions used for blood and urine determinations were found to be stable for several weeks in the refrigerator if made in freshly boiled and cooled glass-distilled water. They were standardized in the usual way against ascorbic acid which in turn was standardized with $N/10$ I_2 solution.

Dosage and blood sampling. Unless stated to the contrary, a standard dose of 700 mg. of ascorbic acid/140 lb. body weight was given orally each day. The dose, in 50 mg. tablets, was administered as nearly as possible in two equal portions, one at the commencement of each 24 hr. collection of urine, the other approximately 12 hr. later. Blood samples were taken, either at the end of the 24 hr. period before the first dose of the subsequent 24 hr., or, if no dose was given, 3 hr. after taking food.

Todhunter, Robbins & McIntosh [1942] showed that after ingestion of synthetic ascorbic acid a maximum rise occurs in the plasma within 1–1½ hr., which may be delayed until 2½ hr. if a natural source of the vitamin, such as cauliflower, is employed. Initially high plasma levels (0·9 mg./100 ml.) may be raised above the level for the renal threshold by a 400 mg. test dose. The effective rise in the plasma level is correspondingly less if a smaller dose is ingested, or the initial level is appreciably lower. These findings justify our division of the standard dose of 700 mg./140 lb. body weight into two approximately equal parts at 12-hourly intervals in order to reduce the sudden overflow through the kidney to a minimum, an event which is more likely to occur the more nearly 'saturated' the subject.

Belser, Hauck & Storvick [1939] and Fincke & Landquist [1942] employed a test dose of 400 mg. and Goldsmith & Ellinger [1939] 300 mg. In general, a 50% excretion of the test dose has been taken as evidence of 'saturation'. Harris took as his criterion the first sharp rise in the urinary excretion, but later modified this to the excretion of 50 mg. or more in the period of 3 hr. commencing 4 hr. after the administration of the test dose. Wright, Lilienfeld & MacLenathen [1937], to avoid disturbance due to faulty absorption, employed a dose of 1000 mg. intravenously, 'saturated' subjects excreting more than 50% in the subsequent 5 hr.

RESULTS

(*a*) *Urinary and plasma ascorbic acid contents during extended saturation tests.* Investigations were carried out on nine subjects to study the behaviour of the plasma ascorbic acid during extended saturation of the subject with ascorbic acid. All subjects took, in addition to the test dose, the normal hospital diet. Evidence is presented elsewhere [Prunty & Vass, 1943] that such a diet is poor in vitamin C. It was thought necessary to establish the fact that the plasma ascorbic acid does not reflect the violent fluctuations which are well known to occur in the urinary output under these conditions, and therefore accurately reflects the intake of ascorbic acid at these levels.

Data typical of our results are given in Table 1 and Fig. 1. The latter brings out the point that the plasma ascorbic acid does in fact remain constant during prolonged saturation, very marked fluctuations in the urinary excretion being shown. Table 1 also indicates the importance of another fact in the interpretation of saturation. During the first period of saturation, 8–16 June 1942 (saturation being demonstrable on the latter date), the rise in the daily urinary excretion was relatively slow and no point of sharp demarcation was obvious. In this case the plasma ascorbic acid at the beginning of the test was 0·00 mg./100 ml., and such a low initial level tended to be associated with this phenomenon in other cases. A second saturation test carried out on the same subject with the plasma level initially at 0·26 mg./100 ml. shows on 15 August 1942 a spectacular rise in the excretion of ascorbic acid, an event easy of interpretation. Furthermore, on attaining saturation some of our subjects did not excrete as much as 50% of the test dose at any time. Therefore we have arbitrarily defined 'saturation' as the excretion of 33⅓% of the test dose based on 700 mg./140 lb. body weight, administered as outlined above.

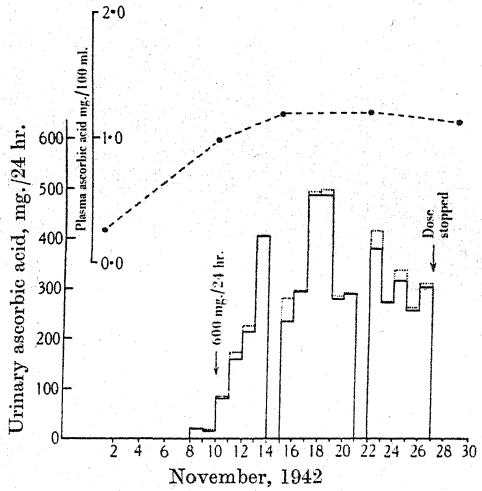

Fig. 1. Saturation test in subject J. Female. Abdominal sinuses. •-------• Plasma ascorbic acid, mg./100 ml. ——— Urine ascorbic acid, mg./24 hr. ------- Urine ascorbic acid + dehydroascorbic acid, mg./24 hr.

The data given in Fig. 1 serve to confirm the fact that the fluctuations in the urinary excretion of ascorbic acid during extended saturation are not due to compensating excretions of dehydroascorbic acid.

(*b*) *The relation of the plasma ascorbic acid to the 'state of saturation' of the subject and resting urinary excretion of 'ascorbic acid'.* The data on this point are compiled in part from that given in the last section and in part from additional data of which Fig. 2 is illustrative. It shows the usual saturation test carried out in six normal subjects with initial plasma levels ranging from 0·31 to 1·46 mg./100 ml. The striking correlation between the time of demonstration of saturation and the initial plasma level is

Table 1. *Extended ascorbic acid saturation test*

Subject B. Male. Gastric ulcer, partial gastrectomy. Body wt. 124 lb.

Date	Ascorbic acid: Dose (mg./day)	Urinary excretion (mg./day)	Percentage of dose excreted	Plasma conc. (mg./100 ml.)	Average urinary conc. over 24 hr. (mg./100 ml.)	Remarks
5. vi. 42	—	—	—	0·00	—	Confined to bed
6. vi. 42	—	16	—	—	0·64	Medical treatment for gastric ulcer
8. vi. 42	600	12	2	—	0·45	
9. vi. 42	600	11	2	0·55	0·50	
10. vi. 42	600	18	3	—	0·73	
11. vi. 42	600	17	3	—	0·65	
12. vi. 42	600	85	14	—	3·68	
13. vi. 42	600	118	20	1·54	3·51	
14. vi. 42	600	—	—	—	—	
15. vi. 42	600	186	31	—	3·36	
16. vi. 42	600	248	41	—	11·42	
17. vi. 42	600	268	45	—	10·05	
18. vi. 42	600	356	60	—	11·18	
19. vi. 42	600	269	45	1·99	11·00	
20. vi. 42	600	329	55	—	19·40	
21. vi. 42	600	—	—	—	—	
22. vi. 42	600	244	41	—	9·20	
23. vi. 42	600	348	58	—	17·60	
24. vi. 42	600	188	31	1·94	15·20	
25. vi. 42	600	267	45	—	11·70	
26. vi. 42	600	244	41	—	11·15	
27. vi. 42	600	257	43	—	11·36	
28. vi. 42	300	—	—	—	—	
29. vi. 42	300	103	34	—	5·64	
30. vi. 42	300	186	62	1·36	10·00	
1. vii. 42	300	88	29	—	5·16	
2. vii. 42	300	129	43	—	6·90	
3. vii. 42	300	134	45	—	8·55	
4. vii. 42	300	48	16	—	2·35	Got up
5. vii. 42	300	—	—	—	—	
6. vii. 42	300	106	35	1·29	5·76	
7. vii. 42	300	95	32	—	11·06	
8. vii. 42 to 17. vii. 42	50 to 50	—	—	—	—	
17. vii. 42 to 23. vii. 42	50 to 50	—	—	1·26	—	
23. vii. 42	50	18	36	—	0·93	
24. vii. 42	50	15	30	—	0·60	
25. vii. 42	50	12	24	0·90	0·62	
27. vii. 42	—	—	—	—	—	Operation
30. vii. 42 to 10. viii. 42	50 to 50	20	40	—	0·86	
10. viii. 42	50	10	20	—	0·66	
11. viii. 42	50	10	20	0·26	0·90	
12. viii. 42	50	18	3	—	0·88	
13. viii. 42	600	33	6	—	2·10	
14. viii. 42	600	70	12	—	5·73	
15. viii. 42	600	421	70	—	23·10	
16. viii. 42	600	460	77	—	32·60	
17. viii. 42 to 6. x. 42	600 to 100	—	—	—	—	Home
	100	—	—	0·62	—	

self-evident. Subject L., with a plasma ascorbic acid of 0·31 mg./100 ml., required 2 days to become saturated, whilst subject V. showed this in one day, the plasma level being initially 0·80 mg./100 ml.

Initial plasma ascorbic acid levels and the corresponding urinary ascorbic acid excretions before saturation with large doses of ascorbic acid, the dosage required to bring to a 'state of saturation', and the corresponding days required to demonstrate saturation are given in Table 2.

Fig. 3 shows the dose required to bring the subject to a 'state of saturation', and the initial plasma level. This dose was computed by determining the doses necessary to produce an excretion of one-third of the test dose (700 mg./140 lb. body weight) in 24 hr. If on a given day the excretion

was less than one-third and on the succeeding day the excretion was greater than one-third, the figure was obtained by interpolation. *From the total dose*

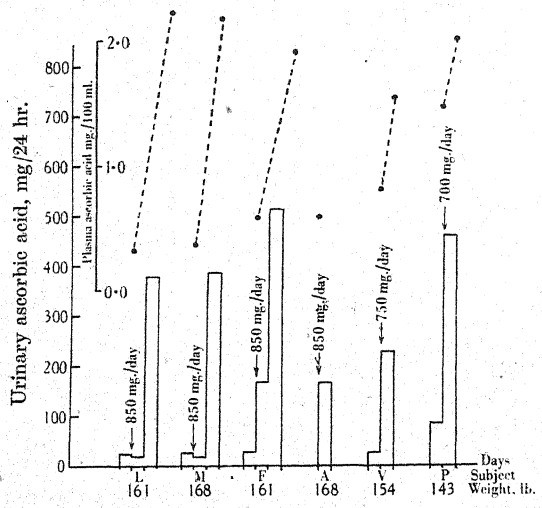

Fig. 2. Plasma level of six normal subjects before and after urinary saturation test. •-------• Plasma ascorbic acid, mg./100 ml. —— Urinary ascorbic acid, mg./24 hr.

are deducted 700 *mg., which represent the single test dose per* 140 *lb. body weight necessary to demonstrate 'saturation' in a subject who has reached a 'state of saturation'*. The point at which the curve crosses the ordinate therefore represents the plasma concentration at the 'saturated state' corresponding to 0·8 mg./100 ml. Thus if the plasma ascorbic acid concentration of the subject, whose saturation is being investigated, is 0·8 mg./100 ml., administration of the test dose results in the excretion of one-third of the test dose. The plasma level reaches or even exceeds the renal threshold, placed by Ralli, Friedman & Sherry [1939] at 1·2–1·4 mg./100 ml.

Table 2. *Initial plasma concentration and daily urinary excretion of ascorbic acid compared with total dosage mg./*140 *lb. body weight, and number of days required to bring subjects to a state of saturation*

	Ascorbic acid			
Subject	Initial plasma conc. (mg./100 ml.)	Initial urinary excretion (mg./day)	Dosage (mg.) required to bring to saturation level/140 lb. body wt.	Days to saturate
S.	0·0	50	4680	8
B.	0·0	24	4200	8
*D.	0·0	18	3620	6
Da.	0·05	24	2600	5
W.	0·08	31	1580	4
Ch.	0·18	13	1700	6
Sa.	0·18	18	1500	4
B.	0·28	13	1580	4
J.	0·26	17	1230	3
L.	0·31	26	580	2
C.	0·33	29	440	2
M.	0·36	24	520	2
Co.	0·56	7	870	3
F.	0·57	27	300	2
A.	0·59	—	230	2
P.	0·76	—	40	1
V.	0·80	27	40	1
Pr.	1·46	85	−430	1

* A case of clinical scurvy.

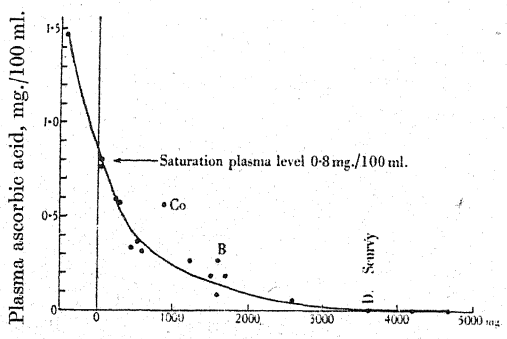

Fig. 3. Initial plasma ascorbic acid concentration against dose to bring to saturation level, with large doses. (700 mg./140 lb. body wt.)

The curve indicates that the relationship between the two variables is not linear, a point that may partially account for the divergence of opinion in the literature as to the validity of 'resting' plasma ascorbic acid concentration as an index of vitamin C nutrition. It must be noted that the only marked deviation from the curve is that of case Co., a child aged 7 years, whereas the other points given are all for adults. The point B. was obtained in the second saturation test (Table 1). The point D. relates to a case of scurvy [Prunty & Vass, 1943].

In spite of the presence of unknown interfering substances, several authorities regard the titre of the 'resting urine' as significant an index as the 'saturation' test itself [Harris, Abbasy, Yudkin & Kelly, 1936; Portnoy & Wilkinson, 1938]. The former stated that 'as under ordinary circumstances the response to a test dose is proportional to the resting level, a measurement of the latter alone is sufficient for most routine tests of the kind here described'. If in an adult 13·8 mg. ascorbic acid are excreted each day, a good response to the test dose of 700 mg./140 lb. body weight is obtained on the first or second day. Table 2 shows that the 'resting' urinary excretion affords little evidence of the status of the vitamin C nutrition of any subject. D., who was clinically frankly scorbutic, excreted as much as the equivalent of 18 mg./day of ascorbic acid, whilst S. and B., who showed no such signs,

both requiring at least 6 days to become saturated, excreted much more than this in the 'resting urine' (see Table 2).

(*c*) *Comparison of the ascorbic acid intake with the maintenance of a known plasma ascorbic acid concentration.* During the course of this work it was possible to maintain only relatively few subjects on controlled intakes of ascorbic acid for prolonged periods during which observations of the plasma ascorbic acid concentration were made. A summary of the results obtained is presented in Table 3. In many cases the greater part of the ascorbic acid ingested was the synthetic product; the remainder of the intake was estimated from a dietetic history [cf. Harris & Olliver, 1943]. The figures fall into two groups, those obtained when the plasma level prior to the period of known intake was lower than the final level and vice versa.

shows that these fluctuations are not due to any interchange between these substances, which is in agreement with the findings of Johnson & Zilva [1934]. Examination of the two saturation tests in subject B. (Table 1) shows the differing urinary excretion responses which are met with in markedly unsaturated subjects (saturation not reached until the sixth day or later) compared with less unsaturated subjects (saturation reached by the fourth day) and illustrates the difficulty, in the earliest urinary saturation test, of noting the first sharp urinary response as an index of the achievement of saturation.

The data given in Table 2 and Fig. 3 establish the fact that the initial plasma ascorbic acid concentration is related to the dose of ascorbic acid required to bring the body to a 'state of saturation', which is attained when the plasma ascorbic acid

Table 3. *The relation between ascorbic acid ingested and the plasma ascorbic acid concentration*

	Ascorbic acid						
Subject	Initial plasma conc. (mg./100 ml.)	Daily dose (mg.)	Estimated dietary intake (mg./day)	Total daily intake (mg.)	Final plasma conc. (mg./100 ml.)	Duration of exp. (days)	Remarks
			A. Plasma concentration previously lower				
P.	0·15	300	50	350	1·52	88	
Ja.	—	150	70	220	1·26	1000	
De.	0·67	200	20	220	1·02	6	Received 75 mg./day for 14 days, 3 weeks prior to this
De.	—	100	20	120	0·67	7	
C.	—	50	50	100	0·33	7	
J.	—	—	70	70	0·26	28	Two oranges daily
			B. Plasma concentration previously higher				
B.	—	100	50	150	0·62	52	Saturated 52 days before
B.	0·90	50	20	70	0·26	38	Dose omitted for 3 days after partial gastrectomy, 3 days after plasma value was 0·90 mg./100 ml.
B.	1·29	50	20	70	1·26	9	
B.	1·26	50	20	70	0·90	21	
Ch.	2·17	50	20	70	1·31	8	Saturated 8 days before
Da.	1·24	50	20	70	1·06	6	Saturated 6 days before
Pr.	1·52	50	100	150	1·46	17	
Pr.	—	—	75	75	0·86	26	Saturated 26 days before
Pr.	0·86	—	50	50	0·58	23	
Pr.	0·58	—	50	50	0·41	23	

DISCUSSION

Our results show that the marked urinary fluctuations met with in the 'saturation' test are not mirrored in the plasma level. This supports the idea that the 'saturated state' is maintained and emphasizes the reliability of the plasma ascorbic acid content as an index of this state. Indeed, Harris [1942], in answer to his critics, considers that these fluctuations do not in any way invalidate the saturation test. An examination of the urinary excretion of dehydroascorbic acid and ascorbic acid reaches 0·8 mg./100 ml. Harris [1942] stated that the necessity of maintaining a state of saturation for optimum nutrition is not established. Ralli & Sherry [1941, p. 290] maintained that in a 'normal' adult the optimal requirement of vitamin C was 100 mg. daily, 'this being the smallest daily dose on which saturation of the body tissues was attained and maintained'. These authors have endeavoured to assess the standard of nutrition in regard to vitamin C by examining the urinary excretion after administration of 100 mg. of the vitamin intravenously. Although they state that in normal (i.e. presumably saturated) subjects 50 % of the injected

vitamin is excreted within 3 hr., 8 out of 12 of their normal subjects failed to satisfy this criterion.

It is widely accepted that the dosage of ascorbic acid required to saturate the subject is a measure of the depletion of the available vitamin C reserves of the body, even though these may be small. It therefore follows from Fig. 3 that the lower the initial plasma ascorbic acid concentration the greater the degree of depletion of the available reserves of the body.

A summary of some of the 'normal' values for the ascorbic acid content of plasma which are to be found in the literature is tabulated below; this range is so great that it is of little clinical value.

Normal plasma values of ascorbic acid, mg./100 ml.

Secher [1942]	Down to 0
Wright, Lilienfeld & MacLenathen [1937]	0·27–1·54
Croft & Snorf [1939]	0·4
Ingalls [1937]	0·5 –2·0
Butler & Cushman [1940]	0·6
Portnoy & Wilkinson [1938]	0·6 –1·85
Abt & Farmer [1938]	0·7 –2·0
Fincke & Landquist [1942]	0·8
Abt, Farmer & Epstein [1936]	0·8 upwards

It is obvious from Fig. 3 that a plasma ascorbic acid value greater than 0·8 mg./100 ml. denotes an intake of ascorbic acid more than sufficient to satisfy the body's requirements and that at values at or near zero the ascorbic acid intake is grossly inadequate. This is borne out by the fact that scurvy does occur when the dosage requirement to bring to saturation is less than that of non-scorbutic subjects (see D., Fig. 3).

Examination of this curve reveals that the maximum change in the dosage of ascorbic acid required to produce a 'state of saturation' at a given plasma level occurs at a value slightly less than 0·4 mg./100 ml. plasma. In view of these facts it is clear that a plasma value of not less than 0·4 mg./100 ml. is compatible with a desirable standard of nutrition with respect to the vitamin. When the initial plasma ascorbic acid concentration is 0·4 mg./100 ml., 450 mg. of ascorbic acid per 140 lb. of body weight will bring the subject to a 'state of saturation' which can be demonstrated by the ingestion of a further 700 mg. of ascorbic acid. This is equivalent to a good saturation response in 2 days with the urinary saturation test of Harris [1943], who states that 'the number of days beyond the second may be taken as an index of the relative deficit in the past intake'.

The data presented in Table 3, although deficient in evidence relating to plasma ascorbic acid levels below 0·26 mg./100 ml., explain certain discrepancies met in the literature. Whereas Bryan, Turner, Huenemann & Lotwin [1941] and Ralli & Sherry [1941] found that a daily intake of 100 mg. of ascorbic acid stabilized the plasma level at approximately 1 mg./100 ml., our results suggest that a daily intake of at least 100 mg. is required to attain a 'state of saturation' with a plasma ascorbic acid content of 0·8 mg./100 ml. Our results also show that if the subject has previously been on a higher intake of ascorbic acid and reverts to a lower one, the plasma ascorbic acid tends to remain at a higher level than that reached when the procedure is reversed, unless a considerable period on the lower intake is maintained (probably at least 30 days). For example, after 21 days on an intake of 70 mg. ascorbic acid daily (subject B), where the initial plasma level was 1·26 mg./100 ml., the plasma level only fell to 0·90 mg./100 ml. This intake, continued for a total of 38 days, gave a final plasma level of 0·26 mg./100 ml. Subject J., on the other hand, who had been receiving the equivalent of 70 mg. daily, had a plasma content of 0·26 mg./100 ml. after 28 days, the dietetic history indicating that the initial plasma level was below that at the end of the test period.

Göthlin [1934], on the basis of his capillary fragility test, suggested at least 19–27 mg. of ascorbic acid daily were necessary to prevent scurvy in the adult. Fox, Dangerfield, Gottlich, Jokl & Suzman [1940] showed that a daily intake of 15–25 mg. of ascorbic acid did not prevent scurvy in a group of Witwatersrand miners. By contrast, Kellie & Zilva [1939] estimated that daily doses of 15 mg. ascorbic acid would suffice to prevent scurvy and maintain good health. They endeavoured to ascertain the minimum daily dose required to maintain 'saturation' and considered this to be achieved when, on a given dose, the ascorbic acid voided in the urine became constant from day to day. Having regard to the overlapping of an increased or decreased daily excretion as the dosage was stepped up or down, they studied the level of excretion reached on ascending from a state of 'unsaturation' and vice versa, with daily doses of 30, 50 and 100 mg. As a result they stated that 'it may be assumed that the minimum dose necessary to bring about "saturation" lies between 30 and 50 mg./day, probably nearer the former figure'. Their interpretation of the results obtained on A.E.K. do not agree with our results on subject B., Table 1, who on 23–25 July 1942 inclusive was excreting diminishing amounts of ascorbic acid in the urine in spite of the fact that the plasma level at the time was 0·9 mg./100 ml. and that B. had been receiving at least 50 mg. ascorbic acid per day for 16 days following a period of 'saturation'. Moreover, the high plasma levels observed in A.E.K. on a continually falling dose agree with our findings in Table 3. Ralli & Sherry [1941] have determined the plasma ascorbic acid values when the subject was on a known intake of vitamin C. With a daily intake of 50 mg., the plasma concentration varied from 0·28 to 0·30 mg./100 ml. when the initial and final plasma concentrations were not appreciably different. When the initial values were 0·97 and 1·45 mg./100 ml. the final values were 0·80 and 0·60 mg./100 ml. (cf. Pr., Table 3, initial value 0·86 mg./100 ml., daily dose 50 mg., value in 23 days 0·58 mg./100 ml., after 46 days 0·41 mg./100 ml.).

On 75 mg. daily intake, when the initial values were 0·3, the final values were 0·5 and 0·7 mg./100 ml.; when the initial value was 1·10 mg./100 ml., the final value was 0·8 mg./100 ml. On 100 mg. daily intake the initial values given are 0·2, 0·5, 0·7, 0·8, 1·25 and the respective final values, 1·07, 1·2, 1·2, 1·12, 1·05 mg./100 ml. These authors computed the daily requirement for an optimal nutrition at 100 mg. of ascorbic acid per day in the adult, by determining the amount retained on a known daily intake, a hazardous procedure in our opinion, in view of the marked

urinary fluctuations they also observed and by reason of the fact that it is not yet possible to determine quantitatively all the metabolic products of ascorbic acid, other than dehydroascorbic acid, which may be eliminated in the urine. Their results also show, as we have observed, that during prolonged saturation the urinary ascorbic acid content tends to fall to a lower value than that initially obtained, which is a further possible source of error in the computation of the ascorbic acid retention.

The most widely accepted figures of the daily requirements for vitamin C in this country and in America are respectively those of The Technical Commission of The League of Nations, Geneva [Bigwood, 1939] (30 mg./day) and The Committee on Food and Nutrition, National Research Council (U.S.A.) [1941] (75 mg./day). Whilst our evidence presented in Table 3 is insufficient a basis on which to dogmatize, it does suggest that at least 70 mg. of ascorbic acid daily are required for a plasma level of the order of 0·4 mg./100 ml. This conclusion, with that of Ralli & Sherry [1941] quoted above, together with the confirmatory observations of Bryan *et al.* [1941] that an intake of approximately 70 mg./day maintained a plasma level of 0·6 mg./100 ml., suggests that at least 60 mg./day are required to maintain a plasma level of as much as 0·4 mg./100 ml. This is in agreement with the view of Bacharach & Drummond [1940] and the Committee on Food and Nutrition (U.S.A.) quoted above, that the requirement of ascorbic acid is 75 mg./day, and that a well-balanced diet should contain this amount.

SUMMARY

1. The plasma ascorbic acid concentration is in man an index of the nutritional state with respect to vitamin C, unless, within the previous 30 days, the subject has been on an appreciably higher intake of ascorbic acid than he is receiving at the time of the test. For clinical purposes this determination is more easily carried out than a urinary saturation test.

2. A plasma ascorbic acid level of 0·8 mg./100 ml. represents a 'state of saturation'.

3. Evidence is presented for the desirability of a plasma ascorbic acid concentration of not less than 0·4 mg./100 ml.

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A Critical Examination of Lugg's Method for the Determination of *l*-Ascorbic Acid

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Recently Lugg [1942 *a*, *b*] has described a procedure designed to increase the specificity of the determination of *l*-ascorbic acid by indophenol titration. The method is based on the differential rates of combination of *l*-ascorbic acid and some other indophenol-reducing substances with HCHO at pH 1·5 and at 3·5. After reaction with HCHO these substances no longer reduce the indicator. In order to ascertain the limitations of this method, Lugg's results have been submitted to a critical examination. In addition we record very briefly some experiments from an investigation which was in progress when Lugg's paper appeared. These were also intended to serve as a basis for a more specific determination of *l*-ascorbic acid and were concerned with the differential properties of the primary oxidation products of *l*-ascorbic acid, reductone and reductic acid. Although the results obtained were less satisfactory than Lugg's, they were not without certain interest.

METHODS

In our investigation we have introduced a few minor modifications which in no way interfered with the general principle enunciated by Lugg. Instead of metaphosphoric acid we employed trichloroacetic acid (final concentration in extract 2·5%). As all our experiments were performed on solutions of pure compounds, the buffers employed were chosen to represent the concentrations of trichloroacetate and citrate which would be present in an extract adjusted to the requisite pH in the course of a determination by the HCHO technique. Also, in carrying out the indophenol titrations, we did not find it necessary to use the 'drop persistence' technique of Lugg. After the direct addition of the indicator the usual correction was made for the amount of dye required to produce the tint observed at the end-point.

Reagents

Formaldehyde—the solutions of formaldehyde were prepared from the Analar reagent by neutralizing the HCl present with NaOH, and diluting with water. The exact concentration was determined by a procedure based on Seyewitz & Gibello's method [1904], or by oxidation with H_2O_2 and titration of the acid formed.

l-*Ascorbic acid*, d-*glucoascorbic acid and reductic acid*—see Snow & Zilva [1941].

Reductone and dihydroxymaleic acid—see Snow & Zilva [1938].

d-*Araboascorbic acid*—kindly presented by Prof. E. L. Hirst. Brownish white crystalline powder. Purity 89% by indophenol titration.

6-*Desoxy*-l-*ascorbic acid*—kindly presented by Prof. T. Reichstein. Pure white crystals. Purity 100% by titration.

6-*Trityl*-l-*ascorbic acid*—prepared by the method of Vargha [1933]. The product prepared by us was similar in characteristics to that obtained by Vargha but its purity as determined by indophenol titration was only 70%.

5:6-*Isopropylene*-l-*ascorbic acid*—prepared by Vargha's method [1932]. Crude product recrystallized four times from acetone. Pure white; m.p. 208° (decomp.). Unlike Vargha, we found that this compound was not readily hydrolyzed in water. Vargha based his conclusion on the fact that in aqueous solution the molecular rotation of the isopropylene compound was identical with that of ascorbic acid. We also found these values to be similar but further work showed that this agreement was fortuitous. Thus when aqueous solutions were diluted with four volumes of ethanol the rotations of ascorbic acid and its acetone derivative were markedly different. It is seen (Table 1) that

Table 1. *Optical rotation of* 5:6-*isopropylene*-l-*ascorbic acid in aqueous and in alcoholic solutions*

(All measurements were made at 21° with light of wavelength 546 mμ.)

Solvent	Time before addition of alcohol to aqueous solution (hr.)	*l*-Ascorbic acid: Concentration (g./100 ml.)	*l*-Ascorbic acid: [α]	5:6-Isopropylene-*l*-ascorbic acid: Concentration (g./100 ml.)	5:6-Isopropylene-*l*-ascorbic acid: [α]
Water	—	1·33	25°	1·63	27°
80% ethanol	0	0·27	52°	0·33	13°
	3½	—	—	0·33	14°
	7	—	—	0·33	16°

even after 7 hr. the change in rotation of the latter was so small that it was probably within the limits of the experimental error. The action of HCHO upon this compound could therefore be studied without any serious interference due to hydrolysis.

Buffer solutions.

	Vol. used to prepare buffers of pH 1·5	Vol. used to prepare buffers of pH 3·5
10% $CCl_3.COOH$ solution	500 ml.	500 ml.
Citric acid (Analar)	24·7 g.	62·5 g.
10N NaOH (Analar)	22·6 ml.	56·8 ml.
Water to	1 l.	1 l.

The pH of each solution was checked electrometrically after addition of 27·5 ml. water and 5 ml. 6M HCHO solution/25 ml. buffer solution. If necessary, small additions of HCl or NaOH were made to the stock solutions to bring the pH after dilution to exactly 1·50 or 3·50.

Procedure. 25 ml. buffer solution and 30 ml. HCHO solution of the requisite concentration, contained in a 100 ml. flask, were placed in a thermostat and allowed to attain the temperature of the bath. As a precaution against atmospheric oxidation a current of O_2-free N_2 was passed through the reaction mixture during the experiment. An aqueous solution of ascorbic acid or other reducing compound (2·5 ml.) was added to make the initial concentration in every case 0·46 mM. Samples of the mixture were then withdrawn at intervals, acidified with HCl to arrest further reaction, and titrated with 2:6-dichlorophenolindophenol. The initial titration value of the reducing compound was determined separately. The results given are in every case the mean of at least two experiments and are expressed as percentages of the original concentration of the reactant.

Special modifications of this procedure were found necessary in the case of certain of the reducing substances employed. The estimation of reductone by direct titration is unsatisfactory because the rate of reduction of indophenol is too slow for the end-point to be easily observed. The reductone solutions were therefore added to an excess of acidified indophenol solution, allowed to stand for 1 min. and back-titrated with ascorbic acid solution stabilized with KCN, NaCl and acid. This method, although not entirely satisfactory in the presence of large concentrations of HCHO, was definitely preferable to the direct titration procedure.

Dihydroxymaleic acid in aqueous solution loses its reducing capacity on standing. In order to avoid this it was dissolved in absolute ethanol before addition to the buffer-HCHO mixture. It was also necessary to dissolve 6-trityl ascorbic acid in ethanol, owing to its low solubility in water.

In the case of alloxantin special precautions had to be taken to avoid oxidation by atmospheric O_2. Water and buffer solutions were boiled and cooled in a stream of N_2 and the reaction was conducted under strictly anaerobic conditions.

RESULTS

The kinetics of the reaction between formaldehyde and l-*ascorbic acid*

The reaction was studied over a range of temperatures and HCHO concentrations. It has been deduced from the data (Table 2) that the reaction is reversible and proceeds according to the equation $A+F \rightleftharpoons AF$ (A = ascorbic acid, F = HCHO, and AF = the complex formed from equimolecular proportions of the reactants). The forward reaction here is bimolecular and the opposing reaction unimolecular. Owing, however, to the high concentration of HCHO relative to that of the ascorbic acid present (23 molar equivalents at the lowest concentration used), the concentration of the former reactant may be considered constant, and the forward reaction therefore behaves as a reaction of the first order, with

$$\frac{dx}{dt} = k_1 (a-x) [F] - k_2 x .$$

From this may be deduced

$$k_1 [F] + k_2 = \frac{1}{t} \ln \frac{x_e}{x_e - x}$$

(a = initial concentration of ascorbic acid; x = amount combined in time t; x_e = amount of ascorbic acid combined at equilibrium; k_1 = velocity constant of forward bimolecular reaction; k_2 = velocity constant of opposing unimolecular reaction; and $[F]$ = concentration of HCHO). From the equilibrium equation the equilibrium constant (K) is obtained as follows:

$$K = \frac{[AF]}{[A]\,[F]} = \frac{k_1}{k_2}.$$

From the experimental results, values for K and $k_1 [F] + k_2$ could be derived, and hence the constants k_1 and k_2 could be calculated.

It will be seen from Table 2 that the figures for K and k_1 at a given temperature were constant within the limits of the experimental error, except for a slight deviation at the highest HCHO concentration at 30°; all values of K for the highest HCHO concentrations, however, must be regarded as somewhat unreliable owing to the smallness of the titration values from which they were derived. The general constancy of K and k_1 demonstrates that the reaction follows the postulated course over a considerable range of HCHO concentrations. An independent check was provided by plotting the values of $k_1 [F] + k_2$ against the HCHO concentration in the case of experiments carried out at 30·3°. The points lay along a straight line intersecting the axis at a point representing the value of k_2. The figure obtained in this way was 0·03, which was in good agreement with the value of 0·028 calculated from the equilibrium constant.

The variation of the velocity constants with temperature agreed with Lugg's results. At a HCHO concentration of 0·526 M, $k_{25°}/k_{15°} = 2·8$ ($k = k_1 [F] + k_2$). Lugg's corresponding figure was 3·0. In the presence of this large excess of HCHO, k_2 was almost negligible and values of

$$\log (k_1 [F] + k_2)$$

plotted against the reciprocal of the absolute temperature were found to lie on a straight line (Fig. 1). The same diagram also shows a similar rectilinear relationship when k_1, k_2 and K are plotted against $1/T$. The change in the equilibrium constant is of practical significance when the method is used under extreme conditions of temperature. The error introduced by temperature changes is much more serious at low HCHO concentrations, and it is probable that Lugg's failure to detect the variation was due to the high HCHO concentration present in his experiments.

Lugg's method is based on the fact that the velocity of combination of HCHO with ascorbic acid is much lower at pH 1·5 than at pH 3·5. In studying the course of the reaction at pH 1·5, only the early stages were considered by us, because of

Table 2. *The influence of temperature on the rate of combination, at* pH 3·5, *of ascorbic acid with varying concentrations of formaldehyde*

Initial ascorbic acid concentration, 0·46 mM. Equilibrium and velocity constants are expressed in mole, litre, minute units.

Temp. (°C.)	HCHO conc. (M)		Uncombined ascorbic acid after T min. (%)										Ascorbic acid combined at equilibrium (%)	Equilibrium constant K	Mean $k_1[F]+k_2$	k_1	$k_2 \times 10^3$
30·3	0·526	T	½	1	1½	2	3	4	6	8	10	20					
		%	54·0	29·3	16·0	8·7	4·1	2·8	2·3	2·0	2·0	1·8	98·0	(92)	1·30	(2·43)	
	0·132	T	2	4	6	8	12	16	20	30							
		%	51·7	28·1	17·1	12·0	8·6	7·4	7·1	7·1	—	—	92·9	100	0·37	2·60	
	0·0526	T	5	10	15	20	25	30	40	50	60	80					
		%	50·8	31·8	22·8	19·7	18·0	17·0	16·9	16·8	16·5	16·8	83·2	94	0·17	2·76	
	0·0263	T	10	20	30	40	50	60	80	100	120						
		%	54·5	37·7	32·1	29·6	28·6	28·4	28·2	28·6	27·8	—	71·8	97	0·099	2·72	
	0·0105	T	5	10	15	20	30	40	60	80	120	150					
		%	86·4	77·2	70·0	63·9	57·4	54·0	50·0	49·1	48·0	47·9	52·1	104	0·058	2·83	
												Mean		99	—	2·73	28
25	0·526	T	1	2	3	4	6	8	12	16	20						
		%	45·5	21·9	10·1	5·8	2·4	2·1	1·6	1·7	1·5	—	98·4	(115)	0·80	1·50	
	0·132	T	1	2	4	8	12	20	30	40	50						
		%	78·4	63·8	43·6	20·5	12·3	6·6	5·4	5·4	5·5	—	94·6	133	0·23	1·64	
	0·0263	T	4	8	16	30	50	80	120	150							
		%	83·2	71·5	54·3	36·6	26·9	23·4	22·8	22·4	—	—	77·6	132	0·057	1·67	
												Mean		133	—	1·60	12
20	0·526	T	1	2	3	4	6	8	12	16	20	30					
		%	62·8	38·4	24·0	15·7	6·9	3·5	2·0	1·5	1·5	1·3	98·5	(125)	0·49	0·92	
	0·132	T	2	4	8	12	16	24	40	60	80	100					
		%	77·3	58·6	38·1	24·5	16·2	9·0	5·1	4·5	4·3	4·3	95·7	171	0·13	0·96	
	0·0263	T	8	16	30	50	92	150	240	360							
		%	80·9	67·8	50·6	36·9	24·0	19·9	18·8	18·7	—	—	81·3	166	0·031	0·96	
												Mean		169	—	0·946	5·6
15	0·526	T	1	2	3	4	6	8	12	20	30	50					
		%	73·2	54·9	43·5	32·8	19·4	11·6	5·0	2·0	1·1	1·0	99·0	(189)	0·29	0·54	
	0·132	T	4	8	16	30	50	80	120	150							
		%	76·1	58·1	34·7	15·2	6·1	3·9	3·4	3·4	—	—	96·6	218	0·071	0·52	
	0·0263	T	30	60	90	120	180	270	390								
		%	70·5	49·9	37·0	28·6	20·3	16·3	15·2	—	—	—	85·3*	220	0·015	0·48	
												Mean		219	—	0·512	2·3
10	0·526	T	1	2	3	4	8	12	20	30	40	90					
		%	81·0	72·0	59·8	51·7	27·7	15·4	5·6	1·5	0·8	0·8	99·2	(247)	0·17	0·31	
	0·132	T	4	8	16	30	50	80	120	150	180						
		%	83·8	70·9	50·5	29·6	14·2	6·4	3·6	2·9	2·9	—	97·1	251	0·044	0·32	
	0·0263	T	30	60	150	240	330	420									
		%	76·7	60·6	32·0	20·1	15·7	13·7	—	—	—	—	87·8*	273	0·010	0·33	
												Mean		262	—	0·323	1·2

* Extrapolated.

(Figures in brackets not included in mean.)

the considerable time required to attain equilibrium. It followed that, at the high concentration of HCHO employed in all the experiments, the quantity of

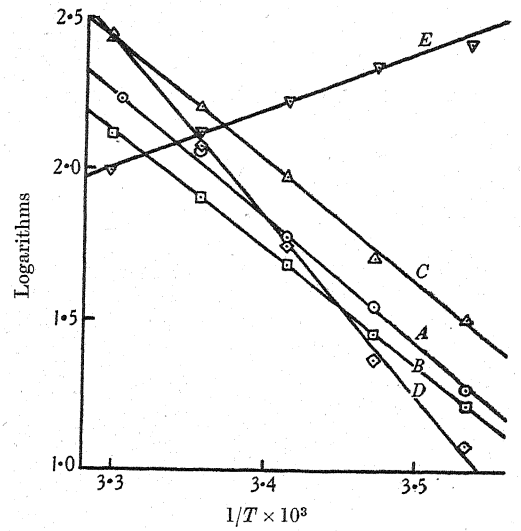

Fig. 1. Reaction between ascorbic acid and formaldehyde. Variation of equilibrium and velocity constants with temperature. (The data were derived from those given in Tables 2 and 3.) T = absolute temp. Curve A: pH, 1·5. $4+\log(k_1[F]+k_2)$. Curve B: pH, 3·5. $2+\log(k_1[F]+k_2)$. Curve C: pH, 3·5. $2+\log k_1$. Curve D: pH, 3·5. $4+\log k_2$. Curve E: pH, 3·5. $\log K$ (equilibrium constant).

ascorbic acid combined at equilibrium could be assumed to be 100 %, without the introduction of a serious error. The calculation was further simplified by neglecting k_2, which is very small under these conditions, and by accepting the expression $k_1[F]+k_2$ as the velocity constant (k) of a first order reaction. The validity of these simplifications was demonstrated in the case of the experiments at 30°, where measurements were continued almost to the point of equilibrium. It will be seen (Table 3) that the amount of ascorbic acid remaining was even smaller than under similar conditions at pH 3·5. When log k was plotted against $1/T$ (Fig. 1), the graph was a straight line with a slope only slightly different from that obtained for the same HCHO concentration at pH 3·5; $k_{25°}/k_{15°}=3{\cdot}1$.

It may be noted that we found a much greater difference between the velocities of reaction at pH 3·5 and 1·5 than that reported by Lugg. He found the velocity constants at pH 1·5 to be 0·03 times those at pH 3·5, whilst the ratio in our experiments was approximately 0·012 (varying slightly with temperature). From the figures given in his paper it would appear that the cause of the discrepancy lies in differences between the velocity constants obtained for the reaction at pH 1·5. The disagreement may possibly be explained by the difference in the composition of the solutions; in our experiments trichloroacetic acid was present, whilst Lugg employed metaphosphoric acid.

The kinetics of the reactions of formaldehyde with substances chemically related to l-*ascorbic acid*

The substances investigated were: *d*-araboascorbic acid, which differs from *l*-ascorbic acid only in the configuration of carbon 4; *d*-glucoascorbic acid, a seven carbon analogue; reductic acid, an alicyclic compound containing a keto-dienol group; reductone and dihydroxymaleic acid, both acyclic compounds with a dienol group, and alloxantin.

d-*Araboascorbic and* d-*glucoascorbic acids.* The velocity of reaction of these two compounds with HCHO (Tables 4 and 5) was similar to that of *l*-ascorbic acid, both at pH 1·5 and 3·5 (cf. Tables 2 and 3). At the higher pH value the reaction rapidly reached an equilibrium, but the

Table 3. *The influence of temperature on the rate of combination of ascorbic acid with formaldehyde at* pH 1·5

Formaldehyde concentration, 0·52M. Initial ascorbic acid concentration, 0·46mM.

Temp. °C.		Uncombined ascorbic acid after T min. (%) / Velocity constant $k=(k_1[F]+k_2)10^3$										
29·7	T	4	8	12	16	20	60	120	180	270	360	
	%	92·9	87·2	82·1	76·4	71·0	35·1	13·3	5·5	2·1	1·5*	
	k	(18·4)	17·3	16·7	17·0	17·4	17·8	(19·4)	17·3	17·1	Mean	17·2
25	T	5	10	15	20	25	30	35				
	%	94·1	89·0	84·1	79·5	75·4	71·3	67·5				
	k	(12·0)	11·7	11·5	11·5	11·3	11·3	11·3			Mean	11·4
20	T	10	20	30	45	50	60	70	80			
	%	93·8	88·3	83·4	76·5	74·5	69·3	65·3	62·4			
	k	(6·45)	6·22	6·07	5·94	5·90	6·11	6·09	5·91		Mean	5·97
15	T	20	40	60	80	100	120					
	%	92·9	86·6	80·5	75·4	70·8	66·2					
	k	3·68	3·57	3·61	3·54	3·46	3·44				Mean	3·55
10	T	30	60	90	120	150	180					
	%	94·6	88·7	85·0	79·8	75·1	70·7					
	k	1·84	2·00	1·82	1·88	1·90	1·93				Mean	1·90

* % ascorbic acid at equilibrium = 1·1 (extrapolated). Figures in brackets not included in mean.

Table 4. *Reaction of various reducing substances with formaldehyde at* pH 3·5

Initial concentration of reducing compounds, 0·46M. Temp. 25°

Reducing compound	HCHO conc. $=[F]$ (M)	Uncombined reducing compound after T min. (%) Velocity constant $k=k_1[F]+k_2$										Reducing compound combined at equilibrium $=x_e$ (%)	$x_e[F]^{-\frac{1}{2}}$
d-Glucoascorbic acid	0·526	T	½	1	2	3	4	8	12	20			
		%	58·9	38·5	15·6	6·0	2·9	0·7	0·5	0·5	—	—	99·5	
		k	(1·07)	0·96	0·94	0·97	0·94	Mean	0·95					
d-Araboascorbic acid	0·526	%	64·2	45·8	24·5	13·2	7·8	3·1	2·4	2·4	—	—	97·6	
		k	(0·92)	(0·81)	0·74	0·73	0·72	Mean	0·73					
Reductic acid		T	½	1	2	4	8	15	30	60	90	120		
	4·8	%	75·4	73·7	73·7	71·7	68·4	65·9	59·9	51·9	47·1	42·9	27	12
	2·4	%	83·6	83·6	84·1	81·1	79·8	78·6	75·1	70·1	67·9	65·9	18·5	12
	1·2	%	89·5	88·0	88·0	87·2	86·7	85·7	84·4	82·9	79·9	78·7	12·5	11
	0·60	%	94·4	93·2	93·2	93·4	92·7	90·9	91·4	90·2	88·9	87·9	8	10
Reductone		T	1	2	4	8	12	16	23					
	4·84	%	77	69	47	26	21	15	11					
		T	10	15	20	25	30	40	60	100	150			
	0·605	%	74	60	—	48	42	34	20	11	7			
	0·151	%	93	88	86	84	79	73	62	46	36			
Dihydroxymaleic acid		T	1	2	4	6	8	12	16	20	30	40		
	4·84	%	91·6	77·5	62·5	48·6	38·5	24·8	15·2	9·9	2·5	0		
		T	2	4	8	12	20	30	40	60	80	100		
	0·605	%	93·7	88·7	80·8	72·2	55·2	41·5	30·9	17·5	10·1	6·0		
		T	6	15	23	30	40	50	60	70	90	110		
	0·151	%	88·8	72·5	63·9	53·2	43·5	35·1	28·5	24·4	15·8	10·9		
		T	15	30	45	60	80	100						
	0	%	78·8	62·2	49·5	37·6	29·0	22·7						
5:6-Isopropylene-*l*-ascorbic acid		T	½	1	2	4	8	15	30	60	90	120		
	0·52	%	78·2	67·4	57·4	51·5	49·5	47·8	44·5	40·1	39·1	37·5	48·5	67
	0·26	%	86·5	80·3	71·1	65·6	62·3	60·4	57·8	54·0	51·0	48·8	36·5	72
	0·13	%	92·0	88·7	83·2	79·1	76·2	74·0	71·2	68·3	64·7	62·3	25	69
	0·065	%	94·0	92·4	89·3	86·2	84·6	82·5	78·7	76·0	74·4	71·6	19	75

(Figures in brackets not included in mean.)

Table 5. *Reaction of various reducing substances with formaldehyde at* pH 1·5

Initial concentration of reducing substances, 0·46mM.

Reducing compound	HCHO conc. (M)	Temp. (°C.)	Uncombined reducing compound after T min. (%) Velocity constant $k=(k_1[F]+k_2)\,10^3$						
d-Glucoascorbic acid	0·52	25	T	5	10	15	20	25	30	
			%	94·2	89·0	84·7	79·2	74·9	69·6	
			k	12·0	11·7	11·1	11·6	11·5	12·1	Mean 11·7
Reductic acid	0·52	30	T	30	60	90	180	270	360	
			%	93·9	90·5	91·2	89·8	88·8	86·1	
Reductone	0·52	30	%	98	93	90	85	74	70	
Dihydroxymaleic acid			T	10	20	30	50	70	90	
	0·52	30	%	73·4	51·5	38·2	21·1	11·4	8·9	
	0		%	79·0	55·7	44·8	24·6	18·6	14·0	

amounts of the reducing substances combined differed somewhat from the corresponding values for *l*-ascorbic acid. The significance of this difference was, however, uncertain in view of the fact that neither of the two compounds was completely pure.

Reductic acid. The reaction of this compound at pH 3·5 was studied over a range of HCHO concentrations. As will be seen from Table 4, there was a rapid initial reaction which was followed by a very much slower one. The two stages can be clearly seen if the titration values are plotted against time. The rapid reaction is then represented by the steep initial part of the curve, the slope of which decreases sharply as this reaction ceases, and the slow reaction is indicated by the lower gradient of the curve as time proceeds. By extrapolating the flat portion of the curve back to the axis it was possible to determine the 'equilibrium' value of the initial reaction. The amount of reductic acid combined at this primary 'equilibrium' was far less than the equilibrium value for ascorbic acid at a corresponding HCHO concentration. The amount of reductic acid combined at 'equilibrium' varied with the HCHO concentration in a manner quite different from that found

with ascorbic acid. Owing to the uncertainty of the nature of the reaction involved it was not possible to calculate an equilibrium constant. An empirical relationship was, however, found to exist between the HCHO concentration and the percentage of reductic acid combined at equilibrium, i.e. $x_e \propto \sqrt{[F]}$ (symbols as in preceding section), as shown by the approximate constancy of the product $x_e [F]^{-\frac{1}{2}}$ (Table 4). The significance of this result is not apparent, and the question of the mode of combination of reductic acid with HCHO remains unsettled. It is, however, evident that at least two reactions are involved, one of which proceeds at a speed comparable with that of the ascorbic acid-HCHO reaction and quickly reaches an equilibrium.

It is probable from the figures given in Table 5 that the course of the reaction at pH 1·5 is quite similar to that at pH 3·5, though the rate of combination is very much slower.

Reductone. Lugg included this substance in the class of compounds which do not condense appreciably with HCHO either at pH 3·5 or 1·5. The results of our experiments at pH 3·5, with different HCHO concentrations, showed, however, that although the velocity of combination of reductone with HCHO was much lower than in the case of ascorbic acid, it was nevertheless significant. This was also found by Mapson [1943]. As the figures for this compound lacked the precision attained with other reducing compounds, the conclusions about the kinetics of this reaction were not so well defined. It was apparent, however (Table 4), that the loss of reductone was approximately exponential and continued until all, or at least a very large part, of this reactant was exhausted. It may be noted that the initial velocity of the reaction was approximately proportional to the concentration of HCHO present. The behaviour of reductone was thus completely different from that of reductic acid. Reductone also reacted with HCHO at pH 1·5, although under these conditions the rate of combination was very slow indeed (Table 5).

Dihydroxymaleic acid. Experiments on this compound were complicated by the instability of aqueous solutions of the substance, both at pH 3·5 and 1·5, even under completely anaerobic conditions, and a comparatively rapid loss of reducing power occurred without the addition of HCHO. The data (Table 4) show that at pH 3·5 the rate of loss of dihydroxymaleic acid in the presence of 0·15M HCHO was only slightly more rapid than in the absence of the latter. Higher concentrations of HCHO, however, caused the reaction to proceed more swiftly, and at a concentration of 4·84M no reducing capacity remained after 40 min. It thus seems possible that, apart from its spontaneous decomposition, the behaviour of dihydroxymaleic acid towards HCHO resembles that of reductone. From Table 5 it will be seen that at pH 1·5 the loss of the compound due to spontaneous decomposition occurred at a rate similar to that observed at pH 3·5, and was only slightly accelerated by HCHO at a concentration of 0·53M.

Alloxantin. Alloxantin combined very rapidly with HCHO both at pH 3·5 and 1·5; at a concentration of 0·52M HCHO, with pH 1·5, no reduction could be detected after 30 sec. at 25°. This compound thus undoubtedly belongs to Class 1 of Lugg's classification, which includes substances that readily condense with HCHO at pH 1·5.

Corrections for certain interfering substances

It is seen from the above that Lugg's method is incapable of distinguishing between *l*-ascorbic acid and its close analogues, and consequently no corrections could be made for their presence. On the other hand, *l*-ascorbic acid can be determined with some degree of accuracy in the presence of high concentrations of reductic acid, reductone or dihydroxymaleic acid if the necessary corrections are made.

In cases where the value obtained after treatment with HCHO at pH 3·5 is small in relation to that obtained by direct titration, no further correction is needed. If, on the other hand, this value be large, corrections for substances behaving like reductone and reductic acid are called for.

If the presence of reductone be suspected, it can be ascertained by oxidizing the extract with indophenol and subsequently reducing it with H_2S. By this treatment only a small fraction of the original reducing value of the reductone is regenerated, whilst the dehydroascorbic acid is completely reduced to ascorbic acid. However, this procedure may not diminish the error due to other substances which behave like reductone in the HCHO technique, and a general correction formula can be introduced in such cases.

The velocity of combination of reductone with HCHO is much lower than that of ascorbic acid at pH 3·5, but in the time required to bring the ascorbic acid reaction to within 0·5% of equilibrium approximately 17% of the reductone would combine. If, however, the experiment is repeated by doubling the reaction time, approximately twice as much reductone will combine, since the course of the reaction is almost linear in the early stages (Fig. 2);

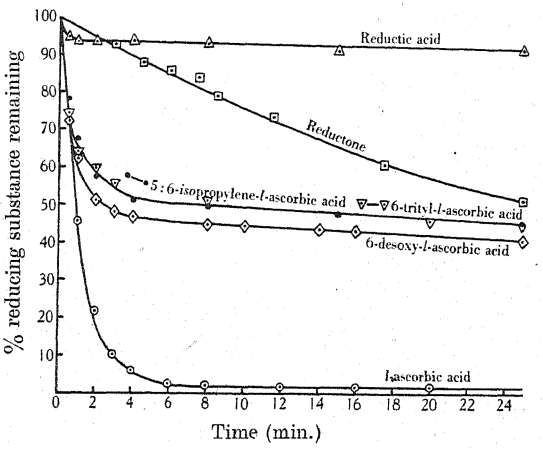

Fig. 2. Reaction of various reducing substances with formaldehyde at pH 3·5. Initial concentration of reducing substances, 0·46mM. HCHO concentration, 0·52M. Temp. 25°.

in both cases the ascorbic acid-HCHO reaction may be considered to have reached equilibrium. Thus the following method of calculation may be employed if reductone or substances behaving like it are present.

$$v_1 = v + w,$$

$$v_2 = v\left(1 - \frac{x_e}{100}\right) + w(1-p),$$

$$v_3 = v\left(1 - \frac{x_e}{100}\right) + w(1-2p),$$

whence $$v = (v_1 - 2v_2 + v_3)\frac{100}{x_e}$$

(v_1 = corrected titration after reaction with HCHO at pH 1·5; v_2 = titration after reaction at pH 3·5 for standard

time (t); v_3 = titration after reaction at pH 3·5 for time $2t$; v = titration equivalent of actual ascorbic acid present; w = titration equivalent of reductone present; x_e = percentage of ascorbic acid combined at equilibrium, and p = fraction of total reductone combined in time t).

This procedure is similar to that adopted by Mapson [1943]. Mapson's modification has the advantage that it does not require special care in the adjustment of the pH and the concentration of HCHO, and is independent of any changes in the reaction velocity at pH 3·5. On the other hand, it requires more material and time for the estimation, and above all, as in the case of Lugg's original method, no correction is made for the error that might arise from the presence of reductic acid.

As the initial velocity of combination of reductic acid with HCHO is comparable with that of ascorbic acid, the procedure recommended for use with reductone and like substances is of no value in this case. However, use may be made of the fact that the amount of reductic acid combined at 'primary equilibrium' is proportional to $\sqrt{[HCHO]}$. After the usual HCHO technique at pH 3·5 is carried out, the procedure is repeated with one-fourth of the concentration of HCHO, the reaction time being suitably increased so as to allow the ascorbic acid-HCHO system to reach equilibrium. In this second titration the percentage of reductic acid combined will be approximately one-half of that in the first titration.

$$v_1 = v + y,$$

$$v_2 = v\left(1 - \frac{x_e}{100}\right) + y(1 - q),$$

$$v_4 = v\left(1 - \frac{x_e'}{100}\right) + y(1 - \tfrac{1}{2}q),$$

whence

$$v = \frac{100\,(v_1 - 2v_4 + v_2)}{2x_e' - x_e}$$

(v_2 = titration after reaction at pH 3·5 with standard HCHO concentration (c); v_4 = titration after reaction at pH 3·5 with HCHO concentration 0·25c; y = titration equivalent of reductic acid present; q = fraction of reductic acid combined at HCHO concentration c; x_e = percentage of ascorbic acid combined at equilibrium with HCHO concentration c; x_e' = percentage of ascorbic acid combined at equilibrium with HCHO concentration 0·25c; v_1 and v have the same significance as before).

Values for the reaction times and the percentage of ascorbic acid combined at equilibrium for the two HCHO concentrations are given in Table 6, in order that this correction may be applied.

The presence of a substance like dihydroxymaleic acid, which decomposes spontaneously both at pH 3·5 and 1·5, would undoubtedly lead to erroneous results in applying Lugg's method, if the extracts were not previously treated with H_2S. If, however, the solutions are allowed to stand in the presence of H_2S, this interfering substance will decompose before the estimation is commenced, and error due to its presence will thus be avoided.

Table 6. *Time required for the combination of ascorbic acid with formaldehyde at* pH 1·5 *and* 3·5

$[F]$ = concentration of HCHO in reaction mixture.
x_e = percentage of ascorbic acid combined at equilibrium.
At pH 1·5, times given are those required to produce the percentage loss indicated.
At pH 3·5, times given are those required to reach 99·5% equilibrium value.

	pH 1·5. Time with $[F]=0{\cdot}52$M		pH 3·5			
			$[F]=0{\cdot}52$M		$[F]=0{\cdot}13$M	
Temp. (° C.)	1% loss (sec.)	5% loss (min.)	Time (min.)	x_e	Time (min.)	x_e
30	35	3	4	98·2	15	93·0
25	60	5	7	98·5	24	94·4
20	100	8·5	11	98·9	40	95·6
15	170	15	19	99·1	68	96·5
10	310	26	32	99·3	119	97·3

(The data are derived from those in Tables 2 and 3.)

The structure of the formaldehyde-ascorbic acid complex

It has already been seen from the kinetics of the reaction, that HCHO and ascorbic acid combine in equimolar proportions. It is therefore probable that the HCHO molecule has two points of attachment in the ascorbic acid molecule. An attempt to isolate the complex, by evaporating concentrated aqueous solutions or by fusing ascorbic acid with polymers of HCHO, did not meet with much success. Rigorous drying gave almost colourless glassy solids which deliquesced immediately on exposure to air and which contained a small amount of unchanged ascorbic acid. When their aqueous solutions were made alkaline and immediately acidified, a large part of the indophenol titration value of the combined ascorbic acid was regenerated. This will be seen later to be characteristic of the complex. Thus these impure solid products contained a considerable proportion of material which behaved in a manner similar to the ascorbic acid-HCHO complex produced in solution. Purification of the solids was rendered difficult by their extremely deliquescent nature, and, in view of the probable complexity of the ascorbic acid-HCHO reaction, further attempts to isolate the complex were felt to be unprofitable. The problem of structure had therefore to be attacked by indirect methods.

The fact that after combination with HCHO the ascorbic acid loses its reducing properties suggests that either or both of the OH groups attached to C_2 and C_3 are involved. Our first object was therefore to inquire into the nature of this linkage. If the condensation were to involve C_3, it should lead to a decrease in the acidic properties of ascorbic acid, since the H atom in this position is probably the one which is mainly ionized [Reichstein, Grüssner & Oppenauer, 1934; Haworth & Hirst, 1934; Haworth, Hirst & Smith, 1934]. In such a case the pH of an unbuffered mixture of HCHO and ascorbic acid should increase as the reaction proceeds. To test the point the following experiment

was carried out. 20 ml. 6M neutral HCHO solution were added to a solution of 0·5 g. ascorbic acid in 180 ml. water at room temperature. The pH of the solution was determined initially and after various intervals by means of a glass electrode system. Simultaneously samples of the mixture were titrated with indophenol. In correlating the pH value and the ascorbic acid concentration of the reacting solution, use was made of a curve previously constructed by measuring the pH of aqueous solutions of known strengths of ascorbic acid. It will be seen from Fig. 3 that although the observed pH values were

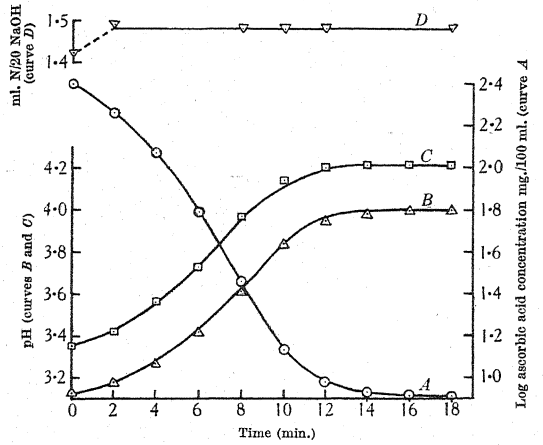

Fig. 3. Changes in pH during reaction between ascorbic acid and formaldehyde. Reaction mixture—0·5 g. ascorbic acid, 180 ml. water, 20 ml. 6M neutral HCHO solution. Temp. 20°. Curve *A*: log ascorbic acid concentration (mg./100 ml.), from indophenol titrations. Curve *B*: pH calculated from ascorbic acid concentration. Curve *C*: pH measured electrometrically. Curve *D*: titration of 5 ml. portions with N/20 NaOH.

somewhat higher than those calculated from the titration values, the changes occurring as the reaction proceeded followed a very similar course in each case. The agreement was quite satisfactory in view of the ease with which the pH of an unbuffered solution may be affected by extraneous substances. The higher pH values were in all probability due to the presence of HCHO. In our opinion this experiment produces sufficient evidence that C_3 is one point of linkage.

In order to determine the position of the second point of attachment, the behaviour of certain derivatives of ascorbic acid containing substituents in positions 5 and 6 was investigated. The compounds employed were 6-trityl-, 6-desoxy- and 5:6-*iso*propylene-*l*-ascorbic acid, and the reaction of each of these substances with HCHO at pH 3·5 was followed up by titration as before. It will be seen (Fig. 2) that the behaviour of the three compounds was remarkably similar, and was in sharp contrast to that of ascorbic acid at the same concentration of HCHO. The course of the reaction was not unlike that observed with reductic acid, though the amount of reactant combined during the initial rapid reaction was much greater at a given HCHO concentration in the case of the 6-substituted compounds. The reaction with 5:6-*iso*propylene-ascorbic acid was investigated over a range of concentrations of HCHO. As in the case of reductic acid the expression $x_e\,[F]^{-\frac{1}{2}}$ was found to be approximately constant (Table 4). Since the behaviour of the three compounds was very similar in spite of the different nature of the substituents, it may be inferred that the contrast between the course of their reactions and that with ascorbic acid was probably not due to any change in the reactivity of the dienol H atoms, but to the blocking of position 6. Also since the 5:6 substituted *iso*propylene derivative behaved exactly like the 6-substituted compounds, linkage at position 5 may be excluded. It thus seems probable that the formation of the HCHO complex involves the 3 and the 6 positions of the ascorbic acid molecule. The possibility that the presence of HCHO might stabilize an abnormal tautomeric form of ascorbic acid having an oxygen bridge attached at C_6 (cf. the structure of *iso*dimethyl ascorbic acid [Micheel & Schulte, 1935; Micheel & Bischoff, 1936; Haworth, Hirst, Smith & Wilson, 1937]) appears to us very unlikely.

In all these experiments it must be emphasized that we have been concerned only with the HCHO condensations which affect the reducing power of ascorbic acid. A few experiments were carried out in an attempt to follow the course of the reaction between ascorbic acid and HCHO by observation of the changes in the optical rotation. The results indicated that the whole reaction system was more complicated than that indicated by titration methods. It is quite probable that other reactions may occur, leading to products which are still capable of reducing indophenol. As the presence of such compounds would have no bearing on the method, the matter was not investigated further.

In connexion with the properties of the ascorbic acid-HCHO complex, we should like to draw attention to a point of interest. In the investigation of the change in pH of the unbuffered ascorbic acid-HCHO solution in the above experiment, samples of the solution were titrated with NaOH (phenolphthalein indicator). Apart from a slight initial rise the titration values remained constant (Fig. 3) throughout the experiment. It would appear from this that the ascorbic acid-HCHO complex is very rapidly decomposed by alkali. This idea was confirmed by the fact that when the alkali-titrated solution was acidified and titrated with indophenol the quantitative regeneration of ascorbic acid was established.

The influence of various substances on the velocity of combination of ascorbic acid and formaldehyde

Since Lugg's method depends upon the rate of combination of ascorbic acid with HCHO at pH 3·5 and 1·5, it is evident that the accuracy of the method would be diminished by the presence of substances capable of influencing the velocity of the reaction. At pH 3·5 the reaction is allowed to reach equilibrium. The times suggested by Lugg are probably greater than is necessary to attain this condition and consequently the possibility of error is reduced. Thus a moderate decrease in the reaction velocity would not be serious, whilst an increase would have no effect upon the result. At pH 1·5, on the other hand, the reaction is allowed to proceed until a definite fraction of the ascorbic acid present (5% in Lugg's precedure) has combined with HCHO, and consequently any variation in the velocity of reaction at this pH would cause some error in the results. This error might be considerable if the reaction were accelerated. We found that when ascorbic acid was added to extracts of certain canned products the rate of reaction with HCHO at pH 1·5 was in fact considerably higher than in pure buffer solutions. Evidently this acceleration was produced by the presence of interfering substances in the extract and we therefore considered it advisable to investigate the influence of some compounds likely to be present in such extracts, as well as that of compounds capable of reacting with HCHO. For this purpose the substances were dissolved in buffer solution containing ascorbic acid and the HCHO solution added when the temperature was stabilized. Care was taken to ensure that the pH of the final mixture was 1·50. Results of these preliminary experiments are given in Fig. 4. It will be seen that, at a concentration of 10mM., any changes in the rate of reaction caused by creatine, *l*-tyrosine, asparagine or leucine were too small to be regarded as significant. On the other hand the presence of glycine (Analar reagent, twice, recrystallized from water), at the same concentration, more than doubled the initial velocity; at higher concentrations the increase was even greater. It must, however, be noted that, whilst the effect with glycine was most marked in the initial stages of the reaction, the increase of velocity observed with the extracts of canned material continued throughout the reaction. The problem requires further investigation, but these results definitely suggest that caution must be exercised in interpreting the results obtained at pH 1·5. If the difference between the direct titration and the values obtained after treatment with HCHO at pH 1·5 is small, no serious error can arise. If, however, the difference is considerable, an inquiry must be instituted in order to ascertain whether this difference is due to the presence of a substance in Class 1 of Lugg's classification or to an increase in the velocity of combination of ascorbic acid with HCHO, brought about by some substance in the extract. It may be noted that many of the substances which are included in Class 1 react almost instantaneously with HCHO at pH 1·5 and therefore the reaction times suggested by Lugg are probably longer than is necessary. We usually allow time for 1% of ascorbic acid to combine instead of 5%. In this way any errors due to changes in the reaction velocity are greatly reduced.

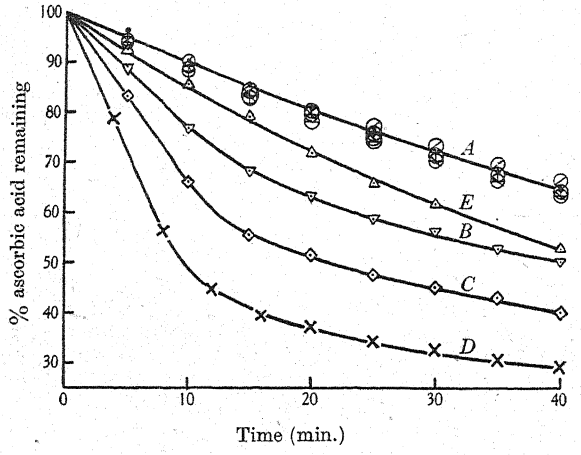

Fig. 4. The influence of various substances on the rate of combination of ascorbic acid with formaldehyde at pH 1·5. Initial concentration of ascorbic acid, 0·46mM. Temp. 25°. HCHO added at zero time. Curve *A*: • No addition; ⓘ 10mM. creatine added; ⊘ 10mM. tyrosine added; ⊘ 10mM. asparagine added; ⊖ 10mM. leucine added. Curve *B*: ▽ 10mM. glycine added. Curve *C*: ◇ 20mM. glycine added. Curve *D*: × 40mM. glycine added. Curve *E*: △ Loss of added ascorbic acid from extract of canned meat and vegetables adjusted to pH 1·5.

The differential behaviour of the oxidation products of ascorbic acid, reductic acid and reductone

It was mentioned in the introduction that at the time of the appearance of Lugg's paper we were engaged in elaborating methods for the determination of ascorbic acid in the presence of reductic acid and reductone. Our experiments were based on our observation that the primary oxidation products of the three compounds possessed certain characteristics which were distinctive enough to enable a separation to be effected. Dehydroreductone could be readily distinguished from dehydroascorbic acid by the fact that only a slight indophenol titration was obtained from the former on treatment with

H_2S at pH 3·5–4·5. Ascorbic acid could therefore be determined, in a mixture of these two compounds, by oxidation and reduction. Reductic acid could be distinguished from ascorbic acid by the greater stability of solutions of dehydroreductic acid at a low pH as compared with those of dehydroascorbic acid, and also because in the oxidized state the former reacted with 2:4-dinitrophenylhydrazine more rapidly than the latter. The following is a short description of the procedure adopted.

(*a*) Sufficient HCl was added to the oxidized solutions to bring the concentration to N/10. The acidified solution was then heated for 1 hr. at 100°, cooled, brought to pH 4·0 by addition of sodium acetate solution, reduced with H_2S and titrated with indophenol, in the usual way, after removal of the excess of H_2S. Under these conditions approximately 89 % of the dehydroascorbic acid and only 3 % of the dehydroreductic acid were destroyed. In practice the accuracy of the method was limited by the tendency for end-points to be indistinct after the acid treatment. Ascorbic acid mixed with 0·1–10M equivalents of reductic acid could be determined with an error not greater than 10 %. For use with tissue extracts it would be necessary to introduce a correction for reductic acid produced, from pectins or pentoses, during the acid treatment, which could be effected by titrating a portion of the solution before reduction with H_2S. It is possible that a more satisfactory procedure could be devised at a lower temperature (40–50°) and higher acid concentration [cf. Penney & Zilva, 1943].

(*b*) The oxidized solution which contained approximately 0·5mM. dehydroascorbic acid or dehydroreductic acid was buffered at pH 1·5. 10 ml. were treated with 2 ml. 0·2 % 2:4-dinitrophenylhydrazine in N HCl, and 1 ml. 2M sodium acetate (to neutralize the acid in the former reagent). The mixture was incubated for 80 min. at 38°, 2 ml. 0·28 % aqueous furfural solution added (to remove excess of the hydrazine reagent), and the incubation continued for a further 10 min. Finally 3 ml. acetate buffer were added to bring the pH to 4·0 and the precipitate removed by filtration. The filtrate was reduced with H_2S and subsequently titrated in the usual way. In the hydrazine treatment approximately 6·5 % of the dehydroascorbic acid and 96 % of the dehydroreductic acid were lost. Ascorbic acid mixed with 0·1–10M equivalents of reductic acid could be determined with an error of less than 4 %. In attempting to apply this procedure to extracts of biological materials we encountered difficulty owing to the formation of colloidal solutions of the dinitrophenylhydrazones.

DISCUSSION

There is little doubt that Lugg's procedure greatly improves the specificity of the indophenol titration for *l*-ascorbic acid. The specificity is, however, not absolute and critical judgement has to be employed in interpreting the results. Apart from the fact that *l*-ascorbic acid cannot be distinguished from its close analogues by this method, every determination must be considered in relation to the circumstances of any given case, in order to correct for the possible presence of interfering substances. These compounds may either belong to a category of compounds capable of reducing indophenol, or may exert an influence on the rate of combination of *l*-ascorbic acid with HCHO.

In applying Lugg's method the following points have therefore to be borne in mind: Lugg assumes that, when equilibrium is reached, 2 % of the ascorbic acid remains uncombined. Our results show that the amount of ascorbic acid at equilibrium depends on temperature (Table 6). This point is of practical importance if the determination is carried out at extreme temperatures. Furthermore, the reaction is very sensitive to slight changes in pH. Although fair accuracy can be obtained under favourable conditions with indicators (methyl orange, thymol blue), the use of a glass electrode system for the control of the pH is desirable. It must also be pointed out that a change in pH may occur on the addition of HCHO. The concentration of HCHO is also important, and the strength of the reagent should be determined before use. It is important, as suggested by Lugg, to pass N_2 through the solutions during the determinations.

Under the conditions employed by us, the times required for the reactions at pH 1·5 and at 3·5 differed somewhat from those given by Lugg. In Table 6 are given the times required for 1 or 5 % of ascorbic acid to combine with HCHO at pH 1·5. At pH 3·5, the times required for the reaction to proceed to 99·5 % of the equilibrium value are given. In practice these times are multiplied by a factor of 1·5 in order to avoid an error due to any slight decrease in the velocity of the reaction that may occur.

Lugg's procedure depends for its accuracy upon the constancy of the velocity of the reaction between ascorbic acid and HCHO. A diminution in the velocity would not be serious unless it were sufficient to prevent the establishment of an equilibrium at pH 3·5 in the time chosen. A considerable acceleration of the reaction, however, would affect the results obtained at pH 1·5. This is a point of practical significance, since it has been found that certain extracts can increase the reaction velocity, and an increase can also be brought about by the addition of glycine. Such errors may be reduced, although not eliminated, by reducing the reaction times employed.

Lugg's method greatly reduces the error in the titration method due to the presence of reductone

or reductic acid, when present in quantities which are small in relation to that of the ascorbic acid present. When larger quantities of these compounds are present, additional corrections must be introduced.

SUMMARY

1. The kinetics of the reaction between HCHO and *l*-ascorbic acid or chemically related substances have been studied.

2. Indirect evidence is produced for the possible structure of the HCHO-*l*-ascorbic acid complex.

3. Lugg's method for determination of *l*-ascorbic acid has been examined from the point of view of the above experiments, and necessary precautions are stressed.

4. Suggestions for other methods for the determination of *l*-ascorbic acid in the presence of reductic acid and reductone are advanced.

One of us (G. A. S.) is indebted to the Medical Research Council for a whole time grant. We should like to express our gratitude to Prof. E. L. Hirst for specimens of *d*-araboascorbic and *d*-glucoascorbic acids, and to Prof. T. Reichstein for a specimen of 6-desoxy-*l*-ascorbic acid. Thanks are also due to Roche Products Ltd. for a gift of *l*-ascorbic acid.

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Studies in Immunochemistry

7. THE ISOLATION FROM HOG GASTRIC MUCIN OF THE POLYSACCHARIDE-AMINO ACID COMPLEX POSSESSING BLOOD GROUP A SPECIFICITY

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Investigations on the nature of the blood group antigens present in human tissues and secretions are greatly restricted owing to the small amounts of active factors present and to the difficulty of obtaining the tissues and secretions in large quantities. An observation of Schiff [1930], however, revealed that commercial peptone contained a considerable quantity of a blood group A factor, and subsequently Schiff & Weiler [1931], Brahn, Schiff & Weinmann [1932] and Schiff [1933] showed that commercial pepsin likewise could serve as a readily available source of this blood group substance. A rich supply of the A-factor was discovered later in hog gastric mucin by Landsteiner & Chase [1936] and by Meyer, Smyth & Palmer [1937]. As a result of their work on the glyco-proteins occurring in gastric mucosa, Meyer *et al.* [1937] isolated from this tissue a polysaccharide that possessed intense A activity and showed that it contained N-acetylglucosamine and galactose in equimolecular quantities. On the basis of the glucosamine content the product was considered to be only 75% pure, and it was observed that the material gave a positive Ehrlich diazo-reaction. Landsteiner & Harte [1940] confirmed these observations and extended the investigation to include a closer examination of the non-hexosamine nitrogenous constituent. They concluded that the A-substance consisted largely of carbohydrate but contained also a component rich in amino-acids.

The A-substance in hog gastric mucin is closely related serologically to the human blood group A factor. In view of the ease with which considerable amounts of hog gastric mucin could be obtained, it seemed a particularly suitable material for experimental study, the results of which could be employed subsequently as a basis for the elaboration of a method for the isolation of the blood group substances from human erythrocytes, tissues and tissue fluids. Cellular and tissue antigens, however,

are usually found to be labile complexes and it is becoming increasingly evident that if their isolation is to be achieved with the minimum of change in the physical, chemical and biological properties, it is essential to avoid conditions that involve extreme pH values, and to work with the material, as far as practicable, at low temperatures. The employment of these restricted conditions for the manipulation of molecular species of biological origin enables certain irreversible changes to be avoided or reduced to a minimum. With the above considerations in mind two methods have been elaborated for the isolation of the specific A-substance from commercial gastric mucin.

Table 1. *The fractionation of an aqueous solution of crude gastric mucin by sodium sulphate precipitation*

Nature of fraction	Amount of fraction* (g.)	$[\alpha]_{5461}$ (c, 0·5 %)	Relative viscosity η	Total N (Kjeldahl) (%)	Ash (%)	Approximate minimum quantity (μg.) inhibiting completely: (a) Isoagglutination	(b) A-haemolysis
(1) Insoluble in water, soluble in alkali	8·6	−45°	1·14†	11·2	3·8	100-200	—
(2) Precipitated from aqueous solution between 27–30 % Na_2SO_4	16·0	+ 8°	2·67	6·1	1·5	0·1	0·01
(3) Soluble in 30 % Na_2SO_4	3·0	−22°	1·22	3·8	3·9	25	2

* From 40 g. of a specimen of crude gastric mucin, of which 23 % was diffusible through a cellophane membrane.
† In 0·05N NaOH.

EXPERIMENTAL

Isolation of the specific A-substance from gastric mucin

Method 1. Commercial gastric mucin, obtained from the Wilson Laboratories, Chicago, was suspended in saline or in water saturated with CO_2. The material, at a concentration of about 2 %, was vigorously shaken and kept saturated with CO_2, until a thick viscous solution was obtained. Usually one or two days were required for the small hard particles of the crude dry gastric mucin to soften, swell and finally pass into solution. The thick, viscous suspension, at a concentration of about 1 %, was centrifuged in the closed bowl of a Sharples centrifuge for 1 hr., and the slightly opalescent supernatant solution was then carefully withdrawn from the bowl by gentle suction. About a third of the original crude gastric mucin remained undissolved. The insoluble material was washed by suspending in saline or in water saturated with CO_2, being washed four times in this manner, and finally dried *in vacuo* (fraction 1, Table 1). The material showed no appreciable A activity as measured by either the haemolysis inhibition or isoagglutination inhibition test. The main aqueous extract, together with the washings after they had been concentrated to small volume, was warmed to about 35°, and 30 % by weight of anhydrous Na_2SO_4 was added, in small portions at a time, to the vigorously stirred solution. The A-substance separated sharply between 27 and 30 % of Na_2SO_4, as a sticky gelatinous mass, and was collected on the centrifuge. The material was suspended in water and after solution was reprecipitated with Na_2SO_4. In some preparations the A-substance was treated with Na_2SO_4 a third time. The final precipitate was dissolved in water, dialyzed at 0° until free from $SO_4^{=}$, spun to remove any insoluble residue, and dried *in vacuo* from the frozen state (fraction 2, Table 1).

Measured by its power to inhibit isoagglutination, fraction 2 is the most active A-substance yet obtained by us. Other constituents (fraction 3, Table 1) of the gastric mucin which were recovered from the Na_2SO_4 supernatant fluids showed much reduced capacity to inhibit the isoagglutination or haemolysis of A-erythrocytes by anti-A serum. The results of fractionating 14·5 g. of fraction 3 from aqueous solution by ethanol are set out in Table 2. The fractions show only slight A activity, which amounts at the most to only a few per cent of the activity of the purified A-substance. This material closely resembles the acid polysaccharide described by Meyer *et al.* [1937]. Further fractionation gave a main fraction with the following properties, $[\alpha]_{5461}$, −34°; N, 3·9 %; limit of inhibition of isoagglutination, 1:20,000.

Table 2. *The fractionation by ethanol precipitation of the* 30 % Na_2SO_4 *soluble material* (14·5 *g.*) *obtained from crude gastric mucin*

Fraction no.	Limits of ethanol concentration for precipitation (% (v/v))	Weight of fraction (g.)	$[\alpha]_{5461}$ (c, 0·5 %)	Relative viscosity η	Total N (%)	Approximate dilution showing complete inhibition of: Isoagglutination	A-haemolysis
1	—	1·2	+36°	1·07	11·1	$1:10^4$	$1:10^5$
2	0–20	1·7	+ 4°	1·08	9·4	$1:10^4$	$1:2\times10^5$
3	20–33	2·7	−14°	1·16	3·8	$1:4\times10^4$	$1:6\times10^5$
4	33–50	3·2	−15°	1·30	4·5	$1:4\times10^4$	$1:10^6$
5	50–66	4·5	−32°	1·19	4·0	$1:8\times10^4$	$1:2\times10^6$

Method 2. Crude gastric mucin was ground in a mortar with 90% phenol. The solvent was added gradually, the material being worked up first to a paste, then to a thick suspension. A total volume of about 2 l. of phenol was used for each 100 g. of gastric mucin, and the final suspension was allowed to stand, with frequent shaking at room temperature, for 24–48 hr. The crude mucin was partly soluble, the insoluble portion being separated from the viscous solution by spinning in the Sharples centrifuge. The insoluble material from each 100 g. of crude mucin was resuspended in about 500 ml. of 90% phenol, allowed to stand for 12 hr., and again recovered from the phenol solution. After a further treatment with phenol this residue was washed with ethanol to remove phenol and dried *in vacuo* (fraction 1, Table 3). The main phenol supernatant fluid, together with the phenol washings from the phenol-insoluble residue, was treated with ethanol to precipitate the A-substance. The ethanol was added as a 1:1 phenol-ethanol mixture in order to avoid local precipitation of the A-substance, which was thrown out of solution as a gelatinous mass when the ethanol concentration reached 10% (v/v). The precipitate was collected, redissolved in half the original volume of 90% phenol, centrifuged to remove any undissolved material and reprecipitated with ethanol. Solution in phenol and reprecipitation with ethanol were both repeated a third time. The final clear, almost colourless jelly was immediately dialyzed at 0°, or was triturated with ethanol to remove excess phenol, and the resulting friable mass then suspended in water and dialyzed at 0° for several days to eliminate the last traces of phenol and ethanol. The clear viscous solution was then frozen at −20° and dried *in vacuo* from the frozen state (fraction 2, Table 3).

The properties of the other fractions obtained by the addition of ethanol to the pooled 10% ethanol solutions are also given in Table 3. The material precipitated from the 10% ethanol-phenol supernatant fluid on raising the ethanol concentration to 25% (v/v) contains an appreciable amount of A-substance which can, however, be readily recovered by repeating the fractionation process. It will be seen that the specific activity, as measured by the capacity of the fractions to inhibit isoagglutination or the haemolysis of sheep cells by anti-A red cell serum, is much less than for the main A-substance fraction (fraction 2), which separated from the 90% phenol solution between 0 and 10% ethanol.

After the preliminary experiments had been completed, two preparations of the A-substance were made by method 1 and three large preparations by method 2. The yields of the final product were in each instance about 33% of the crude gastric mucin and in all the experiments described later in this paper it has not been possible to distinguish between the purified materials isolated by either of the two methods.

A preparation of mucin purchased in this country and purified by the two methods described above yielded purified A-substances that were very similar in properties to the A-substance described in this paper. The yield from the crude mucin was about 50%, the specific rotation of the purified material was, however, slightly less dextrorotatory, $+2° \pm 2°$ and the N was 4·9% or 5·1% according to the method of preparation used. The specimens gave rise to inhibition of isoagglutination at a dilution of $1:1·28 \times 10^6$. The relative viscosity (η, 2·45) was rather less than the value given for the A-substance derived from Wilson's gastric mucin.

Table 3. *The fractionation of crude gastric mucin from* 90% *phenol by ethanol*

Details of fraction	Amount of fraction* (g.)	$[\alpha]_{5461}$	Relative viscosity in 0·9% NaCl†	‡ Total N (%)	Ash (%)	Approximate minimum quantity (μg.) inhibiting completely: (a) Isoagglutination	(b) A-haemolysin
(1) Insoluble in 90% phenol	36·9	+27°§	1·25	6·9‖	14·7	10–100	10–100
(2) Precipitated from 90% phenol by ethanol between 0–10% concentration	38·2	+10°	2·86	5·5	0·9	0·1	0·01
(3) Precipitated from 90% phenol by ethanol between 10–25% ethanol	20·7	− 4°	1·24	8·0	2·0	1·0	0·1
(4) Precipitated from 90% phenol by ethanol between 25–50% ethanol	13·5	−67°	1·14	12·2	2·1	10	20
(5) Soluble in 50% ethanol phenol	14·7	−82°	1·05	10·4	5·2	No appreciable activity	

* From 145 g. of crude gastric mucin, of which, on dialysis, about 20% passed through a cellophane membrane.
† Relative viscosity of 0·9% NaCl taken as 1·00.
‡ Determined by Kjeldahl's method.
§ In 0·1N NaOH.
‖ Uncorrected for ash.

RESULTS

Physical and chemical properties of the A-substance

The material obtained by drying a neutral aqueous solution of the A-substance from the frozen state is of a white, silky, almost crystalline appearance. It dissolves readily in water or saline, and a 1% solution shows considerable viscosity and is faintly opalescent. A 2–3% solution behaves as a viscous solution with pronounced elastic properties, and at a concentration of 4–5% the viscous solution fails to flow out of an inverted test-tube.

Several preparations were examined after solution in buffer acetate (pH 4·0) or phosphate (pH 8·0) buffer (μ, 0·1), in the Tiselius electrophoresis apparatus, by our colleague Dr R. A. Kekwick, who reported (see Addendum) that specimens obtained by either method were essentially homogeneous. Electrometric titration of an aqueous solution of the A-substance failed to reveal any group ionizing between pH 2·0 and 10·5.

The analytical figures for these preparations are recorded in Table 4. The amount of organically bound P was between 0·05 and 0·1 % in all preparations. Sulphur was absent. The acetyl estimations were carried out by the method of Elek & Harte [1936], but, although accurate and reproducible results were obtained with N-acetylglucosamine, the results with A-substance differed by as much as 15 %. The source of error was not detected, although the possibility of acyl residues other than acetyl was considered. Acetic acid was isolated as Ag salt (found, Ag 64·6; calc. 64·7 %) from an acid distillate of A-substance. The yield was of the same order (75 %) as was obtained in similar estimations on acetic acid.

Table 4. *The analytical data for preparations of the A-substance isolated from gastric mucin*

Method of preparation and no.	Weight of preparation (g.)	$[\alpha]_{5461}$ (0·5 % in H_2O)	Relative viscosity (0·5 % in saline)	Content (%) of C	H	N*	Ash	Acetyl
Phenol extraction								
GM. 201	30	+11°	2·80	—	—	5·7	1·4	9·6
GM. 210	33	+14°	2·86	45·2	6·6	5·9	0·9	10·7
GM. 240	38	+10°	2·83	45·1	6·7	6·0	0·9	10·3
Aqueous extraction								
GM. 280	9	+ 8°	2·78	44·8	6·4	6·4	0·8	9·2
GM. 321	20	+12°	2·35	45·9	6·4	6·2	0·6	11·5

* By Dumas's method.

Viscosity. The relative viscosity values of 0·5 % solutions of the preparations in 0·9 % NaCl were determined at 37° by means of a modified Ostwald viscosimeter. For the three large preparations of A-substance extracted by means of phenol, the figures were 2·80, 2·86, 2·83, whereas two preparations, isolated by means of aqueous extraction, gave values of 2·78 and 2·35; the viscosity of 0·90 % NaCl being taken as 1·00. After heating with 0·1 N acetic acid at 95° for 10 min., with removal of the acid by dialysis, and recovery of the product by drying *in vacuo* from the frozen state, the A-substance, dissolved in 0·90 % NaCl, showed a relative viscosity of 2·07. Material which had been treated with 2 % Na_2CO_3 at 70° for 15 min. [Meyer *et al.* 1937], and likewise recovered after dialysis, also showed a considerably decreased viscosity (η, 1·27) and treatment of the undegraded high viscosity A-substance with anhydrous formamide at 150° for 1 hr., according to the technique used by Landsteiner & Harte [1940], reduced the relative viscosity of the A-substance to 1·09, a figure only slightly higher than that for 0·90 % saline. In view of the ease with which the viscosity of the A-preparations was destroyed it was considered of interest to investigate the influence, on viscosity and serological activity, of heating the material dissolved in buffered saline at pH 3·0, 5·0, 6·9 and 8·0. The change in viscosity with time of heating an 0·5 % solution at different pH values is shown in Fig. 1. The solutions were sealed in ampoules before heating. The rapidity with which the viscosity is lost when the A-substance is heated on the alkaline side of neutrality (pH 8·0) is of interest and indicates that the A-substance should on no account be heated at alkaline reaction. It is clear that on the acid side of neutrality the A-substance is definitely more stable, although considerable loss in viscosity takes

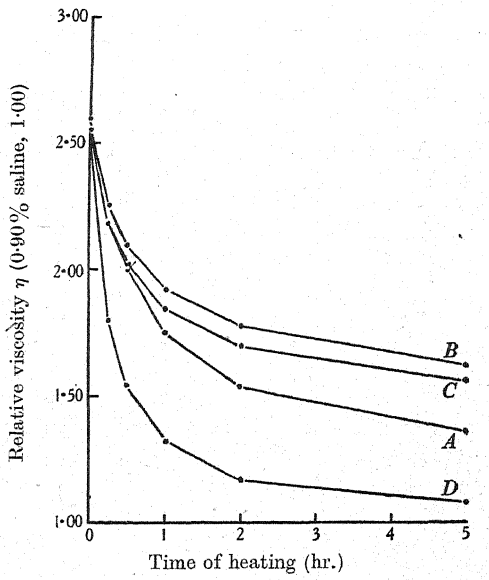

Fig. 1. Curves showing the fall in viscosity of undegraded A-substance on heating at 100° in buffered saline at pH 3·0, *A*; pH 5·0, *B*; pH 6·9, *C*; pH 8·0, *D*.

place on prolonged heating, even at pH 5·0 or 6·9. It is evident that such treatments induce a greater or lesser degree of physical degradation according to the conditions selected. The profound influence of some of these methods of treatment on the capacity of the A-substance to inhibit isoagglutination is shown in Table 5 and is discussed later.

An aqueous solution of the A-substance does not precipitate when treated with an equal volume of 2 % solutions of the following reagents: picric acid, trichloroacetic acid (10 %), aluminium sulphate, phosphotungstic acid (with or without 5 % H_2SO_4), lead acetate, ferric chloride, potash alum, Fehling's solution and silver nitrate. The addition of 0·01M mercuric acetate, however, causes immediate gelation. Similarly, the addition of an equal volume of 0·05M borate buffer (pH 8·5) to a 1·0 % solution of the A-substance gives rise to gel formation; the system, however, still retains pronounced elastic properties. The elastic quality may be readily shown by rotating a thin gelatinous solution in a suitable vessel and observing the return motion that follows immediately the original rotational motion has stopped. Air bubbles rising through a gel prepared in this manner from a 1 % solution assume a pear shape, the tail of each bubble remaining attached to the bottom of the container and becoming long and thin as the bubble slowly rises to the surface. The sol-gel change is reversible. Dialysis of the gel at 0° against distilled water leads to the formation of the original viscous solution; the addition of borate buffer will again give rise to a firm, elastic gel. These reversible sol-gel changes do not occur with degraded preparations of the A-substance.

Chemical composition. The A-substance gives no positive colour test when treated directly with *p*-dimethylamino-benzaldehyde but gives rise to a strong purple colour if previously heated at 100° with 0·05N Na_2CO_3 for 6 min., according to the method of Morgan & Elson [1934]. The colour developed is equivalent to that given by a content of 12–13 % of N-acetylglucosamine. This high value was difficult to explain in view of the fact that the preparations of A-substance showed only 0·4–0·6 % of reduction by Somogyi's [1926] copper reduction method. In order to determine the N-acetylhexosamine colorimetrically, however, the A-substance must be heated with 0·05N Na_2CO_3 and it was found that during this treatment the reducing power of the substance increased rapidly to a value between 3·0 and 3·5 % for different preparations. Heating for 6–30 min. gave rise to a maximum figure of 3·6 % reduction. The reducing power of N-acetylglucosamine, determined by the above method, is equivalent to 27 % of an equal weight of glucose; therefore, if we assume the whole of the reduction (3·3 % average value) is due to N-acetylglucosamine, then the N-acetylglucosamine colour should be equivalent to 12·2 % N-acetylglucosamine, whereas values between 12 and 13 % were found for different preparations. Several complex polysaccharide substances that were known to contain hexosamine units, such as hyaluronic acid, chondroitinsulphuric acid and the O somatic antigens of *Shigella dysenteriae* and *Eberth. typhosa*, as well as the simple methylhexosides, N-acetyl- and N-benzoylmethyl glucosamides, failed to give any colour when tested with Ehrlich's reagent after heating with 0·05N Na_2CO_3. These results suggest that within the A-polysaccharide complex there exists an alkali-labile glycoside linkage involving the first carbon atom of the N-acetylhexosamine component. We have observed that the reddish purple colour develops with the A-polysaccharide rather more slowly than with N-acetylglucosamine; colorimetric readings should, therefore, be taken at the end of 90 min., rather than after 45 min. as originally suggested. An identical colour reaction, representing 10–11 % N-acetylglucosamine, is given by the formamide-degraded A-substance.

The immunological properties of the A-substance

A measure of the distribution of the blood group A-substance throughout the different fractions arising during the process of isolation was obtained by means of the ability of the A-factor to (1) inhibit isoagglutination and (2) prevent the haemolysis of sheep erythrocytes by an anti-A rabbit immune serum.

The isoagglutination inhibition test was carried out as follows. Diminishing concentrations of the preparation under test (0·1 ml.) were mixed with 0·1 ml. of a human anti-A (α)-serum, representing in amount 3–4 completely agglutinating doses, and allowed to stand at room temperature for 1 hr. Freshly drawn A-erythrocytes (0·5 % in 0·9 % NaCl) were then added (0·1 ml.). The mixtures were thoroughly shaken, set aside for 2 hr. at room temperature and then examined for isoagglutination. The dilution of the A-preparation allowing minimal agglutination—that is, a few groups of 2–3 cells present in an otherwise unagglutinated microscopic field—was taken as the end-point of inhibition. The sterile anti-A serum, used as a standard α-agglutinin reagent throughout the investigation, was stored without antiseptic in sealed ampoules at −20°. The serum was tested from time to time against the erythrocytes of four group A_1 persons of average sensitivity, working in the laboratory; the agglutination end-point remained constant, throughout the investigation, at a dilution of 1:192. An additional check on the inhibition test was obtained by including a 'standard' purified gastric mucin preparation in each batch of tests; the inhibition titre obtained with this material remained unchanged over a period of 2 years.

The haemolysis test was made as follows. Dilutions of the gastric mucin (0·5 ml.) were mixed with 2–3 minimal haemolytic doses (M.H.D.) of anti-A rabbit immune serum (0·5 ml.), and 0·5 ml. of 1:10 dilution of fresh guinea-pig complement was added to each tube. The mixture was incubated at 37° for 1 hr. and 0·5 ml. of a 4 % suspension

Table 5. *The inhibition of A-isoagglutination by different preparations of A-substance obtained from gastric mucin*

A-substance prepared by	Subsequent treatment	Degree of isoagglutination. Dilution* of A-preparation used in test: 1 : 10^4	1 : 2 × 10^4	1 : 4 × 10^4	1 : 8 × 10^4	1 : 1·6 × 10^5	1 : 3·2 × 10^5	1 : 6·4 × 10^5	1 : 1·28 × 10^6	1 : 2·56 × 10^6	Control
Method 1 (water or saline extraction and Na_2SO_4 fractionation)	Untreated	0	0	0	0	0	0	1	1	2	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	1	2	2	3	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	1	2	3	3	
	Fractionated between 90 and 92% acetic acid	0	0	0	0	0	0	1	2	2	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	1	1	2	2	3	4	4	
Method 2 (phenol extraction)	Untreated	0	0	0	0	0	0	0	1	2	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	2	2	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	1	1	2	3	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	0	1	1	2	3	3	—	
	Heated in formamide, 1 hr. at 150°	0	1	2	2	3	3	3	3	—	
Material from crude gastric mucin insoluble in 90% phenol	None	1	2	4	4	4	4	4	—	—	
Protein fraction of crude gastric mucin soluble in 50% ethanol-phenol	None	4	4	4	4	4	4	4	—	—	

* Final dilution in the tubes is three times the dilution given in the table.

Table 6. *The inhibition of the haemolysis of sheep cells in* 2–3 M.H.D. *of anti-A rabbit serum by preparations of A-substance*

A-substance prepared by	Subsequent treatment	Degree of haemolysis. Dilution* of A-material used in test: 1 : 10^5	1 : 10^6	1 : 2 × 10^6	1 : 4 × 10^6	1 : 8 × 10^6	1 : 16 × 10^6	1 : 32 × 10^6	1 : 64 × 10^6	1 : 128 × 10^6	Control
Method 1 (water or saline extraction and Na_2SO_4 fractionation)	Untreated	0	0	0	0	0	0	1	2	4	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	1	2	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	0	1	2	4	
	Fractionated between 90 and 92% acetic acid	0	0	0	0	0	0	0	1	3	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	0	0	0	0	1	2	3	
Method 2 (phenol extraction)	Untreated	0	0	0	0	0	0	1	2	3	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	2	2	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	0	0	1	2	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	0	0	0	0	1	2	3	
	Heated in formamide, 1 hr. at 150°	0	0	0	0	0	0	1	2	4	
Material from crude gastric mucin insoluble in 90% phenol	None	4	4	4	4	4	4	4	4	4	
Protein fraction of crude gastric mucin soluble in 50% ethanol-phenol	None	4	4	4	4	4	4	4	4	4	

* Final dilution in the tubes is four times the dilution given in the table.

of washed sheep red cells was added. The greatest dilution of the A-preparation allowing just visible haemolysis to occur was taken as the end-point of the haemolysis inhibition. The control tube containing saline in place of the gastric mucin dilution showed complete haemolysis in 30 min. Because of the variable nature of the haemolytic system all the results given in Table 6 were obtained in a single experiment and are therefore strictly comparable.

The results of titrations by both serological tests are given in Tables 5 and 6. The end-point of inhibition of isoagglutination given by preparation GM. 280, which was prepared by aqueous extraction of the crude mucin (method 1), is not significantly different from that given by preparation GM. 240, which was obtained by the use of 90% phenol (method 2). The first traces of isoagglutination appear in the tube containing 0·1 ml. of a $1:6{\cdot}4 \times 10^5$ (GM. 240) or $1:1{\cdot}28 \times 10^6$ (GM. 280) dilution of the A-preparation. The absolute amount of the A-substance present is about 0·1 μg. It will be observed from the results (Table 5), however, that heating these preparations in neutral saline at 100° for 120 min. causes a decrease (50–75%) in the power of the preparations to inhibit isoagglutination. Similarly, treatment with 2% Na_2CO_3 at 70° for 15 min. reduces considerably the activity of the preparation, and treatment with formamide at 150° for 1 hr. destroys more than 95% of the power of the preparation to inhibit isoagglutination. A 1% solution of the A-substance in 0·05N Na_2CO_3 shows less than 1% of the original activity (inhibition of isoagglutination) after heating at 100° for 15 min. After heating at pH 3·0, 6·9, 5·0 and 8·0 in sealed ampoules for 5 hr. at 100°, the capacity of the A-polysaccharide to inhibit isoagglutination fell to 12, 25, 25 and 2% respectively of the original value.

When the serological activity of the A-preparation is measured by the haemolysis inhibition test, however, a different picture is presented (Table 6). Again the serological activity of the undegraded material prepared by either method appears to be the same, and the absolute amount of A-substance, giving rise to almost complete inhibition of haemolysis when mixed with 2–3 M.H.D. of an anti-A rabbit serum prepared against human erythrocytes, is about 0·01 μg. Treatment with 2% Na_2CO_3 at 70° for 15 min., with formamide at 150° for 1 hr. or with neutral saline at 100° for several hours, however, causes no destruction of the capacity of the preparation to inhibit haemolysis. Indeed, it will be seen that a slight increase in activity occurs on heating the A-substance at 100° for 2 hr. An increase in activity of this order for formamide-treated material has already been reported by Landsteiner & Harte [1940]. The use of an anti-serum (rabbit) to gastric mucin (2–3 M.H.D.), in place of the anti-A erythrocyte serum gave similar results [Morgan, 1943].

A specimen of the purest preparation of A-substance was tested for antigenicity in rabbits, 0·1 or 1·0 mg. being injected intravenously at 3–4 day intervals. After three or six doses, serum samples were collected 7 days after the last dose and titrated for anti-A immune body by means of the agglutination reaction with fresh human A-erythrocytes. In no instance was any significant rise in anti-A (α)-agglutinins recorded. An account of the immunological properties of the purified A-substance is given in greater detail elsewhere. It will suffice here to state that the conversion of the A-substance into a full antigen possessing A specificity has already been accomplished [Morgan, 1941; 1943].

Attempts to remove the amino-acid-containing components from the A-substance

In some respects the chemical nature of the A-substance resembles that of the O antigens of certain gram-negative bacteria. These biologically important complexes contain a large proportion of polysaccharide, a conjugated protein component making up the remaining part of the structure essential for the manifestation of antigenicity. Earlier work [Morgan & Partridge, 1940; 1941; 1942; Partridge & Morgan, 1940] on the nature of these substances has shown that in certain instances the bacterial antigenic complex can be dissociated into its component molecules by special means, and this knowledge suggested that the A-substance should be submitted to similar procedures with the hope of attaining a complete or partial dissociation of the component molecules. The methods employed and the results obtained are briefly described.

Fractionation from phenol solution. A-substance (2·0 g.) was treated with 250 ml. of 90% phenol, allowed to stand for 24 hr. and spun for 1 hr. in a Sharples centrifuge. The thin gelatinous substance which collected on the walls of the centrifuge bowl, and which represented the portion of the A-substance that was less readily soluble in phenol, was washed with ethanol to remove phenol, and dried. It weighed 0·83 g., possessed $[\alpha]_{5461} + 13°$, contained 5·8% N and showed a relative viscosity in saline of 2·85. The dilution that showed almost complete inhibition of isoagglutination was $1:6{\cdot}4 \times 10^5$. The phenol supernatant fluid was dialyzed until free from phenol and evaporated *in vacuo* to dryness. The material recovered weighed 0·56 g., showed $[\alpha]_{5461} + 8°$, contained 5·4% N and was only slightly less active than the original material in inhibiting isoagglutination. Relative viscosity, η, 1·59.

It has been observed that dry preparations of the purified A-substance prepared by either of the methods described are never completely soluble in 90% phenol, although originally the material was readily soluble in liquid phenol of this strength and could be repeatedly dissolved in the solvent and precipitated by the addition of 10% ethanol. The fractional extraction of the purified and dry A-

substance with 90% phenol indicates that the preparation is most probably chemically homogeneous, but is certainly not homogeneous with regard to particle size. The high viscosity of the phenol-insoluble portion when dissolved in neutral saline suggests that the A-substance is present as long chain aggregates of considerable molecular size, whereas the low viscosity of the phenol soluble portion indicates that this material consists of depolymerized aggregates having smaller chain lengths in aqueous solution.

Fractionation from formamide solution. The A-preparation (3·0 g.) was dissolved in 125 ml. of anhydrous formamide, cooled to 0° and treated with an equal volume of ethanol previously cooled to −20°. No precipitate was formed up to 45% (v/v) ethanol but a considerable amount of material separated when the ethanol concentration was increased to 50%. The preparation was collected and the material remaining in the ethanol-formamide was recovered by dialysis and evaporation. The substance thrown out of solution by ethanol was again dissolved in formamide, and the precipitation repeated. The thrice precipitated material weighed 1·1 g., showed $[\alpha]_{5461}+16°$, contained 5·4% N and was active in inhibiting isoagglutination at a dilution of $1:6{\cdot}4\times10^5$; η, 2·87; ash, 0·4%. The material recovered from the combined ethanol-formamide solutions weighed 1·07 g., showed $[\alpha]_{5461}+18°$; N, 5·7%; η, 1·57; and was, by both serological tests, as active as the original material.

Fractionation from aqueous solution by organic solvents. A 1·6% solution of the A-preparation was used in each experiment. Treatment with acetone gave rise to two fractions, collected over the ranges 39–41% (v/v) and 41–50% (v/v) of acetone. Practically the whole of the material was precipitated by ethanol between the levels 47–50% (v/v) and by acetic acid between 90 and 92% (v/v). In each instance, however, the precipitated products possessed the same specific rotation, N content and serological titre as the original material.

Fractionation with ammonium sulphate. A-substance (5 g.), dissolved in 300 ml. water, was fractionated by addition of solid ammonium sulphate. Up to 72% of saturation, only a small amount of material separated, and this appeared to be similar to the original preparation. The main bulk (2·74 g.) of the A-substance was precipitated on raising the ammonium sulphate concentration to 81% of saturation. The material was collected, dialyzed free from the salt, precipitated with ethanol and dried *in vacuo*. The properties, $[\alpha]_{5461}+13°$; N, 5·4%, of this fraction were substantially the same as those of the original material. Removal of the salt from the 81% saturated supernatant fluid by dialysis and recovery of the material, however, gave rise to 0·62 g. of material, which was of low serological activity as measured by the isoagglutination inhibition test. The substance showed, $[\alpha]_{5461}-14°$, and possessed a relatively low N content (4·6%). The main fraction (2·74 g.) was redissolved in water and again fractionated as above, yielding a further 0·12 g. of similar material showing *laevo* rotation and low N. A third fractionation of the main fraction gave an additional small quantity of material showing similar properties, $[\alpha]_{5461}-5°$ N, 4·3%, and possessing very low activity measured according to the isoagglutination and isohaemolysis inhibition tests. At first it was thought that these fractions, which were readily soluble in high concentrations of ammonium sulphate, and differed sharply from the original A-substance in chemical and serological properties, might represent the partly separated polysaccharide component of the A-substance. No definite evidence for this conclusion has, however, been obtained.

The above experiment yielded a highly purified sample of the A-substance. This material, thrice fractionated with ammonium sulphate, showed the following properties; $[\alpha]_{5461}+12°$; N, 5·63%; η, 2·59: maximum dilutions showing almost complete inhibition of isoagglutination, $1:6{\cdot}4\times10^5$, and of haemolysis $1:32\times10^6$, respectively.

Fractionation with ammonium sulphate from alkaline solution. The technique was similar to that described above, but after each addition of the salt NaOH was added to maintain alkalinity. Only a single fractionation was performed. The results were similar to those obtained with the neutral solution of the material described above. The small fraction (5%) soluble in high concentrations of the salt showed the following properties $[\alpha]_{5461}-27°$; N, 4·6%. The activity of this material, as measured by both serological tests, showed only a few per cent of the original value.

Fractionation after heating with acetic acid. The possibility of separating the amino-acid component from the A-substance after degradation of the complex by heating with dilute acetic acid was investigated. A 1·6% solution (100 ml.) was heated at 100° with 1% acetic acid for 2 hr. The opalescence of the solution increased until it became quite opaque, but no material separated from solution. Without neutralization of the acid the solution was treated with ammonium sulphate. The first and main fraction was thrown out of solution between 70 and 85% saturation with the salt. This material separated, during dialysis, into a water-soluble and a water-insoluble part; the latter fraction, however, dissolved readily on the addition of a little Na_2CO_3. The water insoluble-material weighed 0·55 g., showed $[\alpha]_{5461}+9°$, contained 5·9% N and was as active in the inhibition of haemolysis as the original material. The water-soluble fraction showed a lower relative viscosity (η, 1·37) compared with the original unheated material (η, 2·83), the rotation was somewhat higher, $[\alpha]_{5461}+20°$, and the N content was unchanged (5·6%). This material, apart from being degraded by treatment with hot acetic acid, was essentially unchanged chemically, but the capacity to inhibit isoagglutination was only about one-quarter of the original value. Once again the small fraction of the original material soluble in high concentrations of ammonium sulphate (above 85% saturation) showed a *laevo* rotation, $[\alpha]_{5461}-20°$, a somewhat lower N (4·9%) and was only weakly active in the isoagglutination and haemolysis inhibition tests.

The action of trypsin. A purified preparation of gastric mucin A-substance (1 g.) was dissolved in 100 ml. of 15% urea solution and the pH was adjusted to about 9·0 with Na_2CO_3. Commercial trypsin (100 mg.) was then added and the mixture incubated at 37° for 6 hr. The solution was then dialyzed at 0° to remove urea, and concentrated to about 10 ml. Solid ammonium sulphate was added with thorough mixing and three fractions were removed. A small amount of material (45 mg.) separated on the addition of ammonium sulphate up to 72% of saturation. The material contained 5·5% N but was not clear enough for polarimetric examination even after the addition of alkali. The second and main fraction (0·62 g.), separating between 72 and 79% saturation, contained 5·6% N and showed $[\alpha]_{5461}+12°$. A small amount of material (60 mg. N, 4·7%) separated between 79% and full saturation at 18°, and a

further 65 mg. of material was finally recovered from the saturated ammonium sulphate solution. All fractions, with the exception of the last (not tested), showed no very significant change in serological activity. The treated material, however, had a lower viscosity (2·17) than the original preparation.

Treatment with chloroform-amyl alcohol. A 2% solution of A-substance ($[\alpha]_{5461}+12°$; N, 5·70%) was shaken with an equal volume of $CHCl_3$-amyl alcohol mixture according to the method of Sevag [1934]. The aqueous layer, after several treatments with the $CHCl_3$-amyl alcohol mixture, was fractionated with ammonium sulphate. The main fraction, 63% of the original material, separated between 73 and 82% of salt, showed a specific rotation $[\alpha]_{5461}+12°$ and contained 5·25% N. An additional 10% was recovered from the supernatant fluid. This fraction showed a lower N content but was otherwise similar to the main fraction. Both materials gave inhibition of isoagglutination at a dilution of $1:6{\cdot}4\times10^5$.

It would appear that the application of the techniques described have in no instance succeeded in removing the amino-acid components from the polysaccharide moiety of the A-substance. The application of certain of these procedures, however, removes about 5% of material which shows a *laevo* rotation and possesses a lower N and a considerably diminished specific activity when compared with the purified A-substance.

Acid hydrolysis of the A-substance

The A-substance is readily hydrolyzed by heating in N HCl at 100°. Complete liberation of the component amino-acids, however, is only attained after hydrolysis with 6N HCl. The hydrolysis was carried out in sealed ampoules, samples being removed from time to time and immediately neutralized. during hydrolysis was followed by Van Slyke's method [1929] and it is to be noted that any free amino-groups liberated through the elimination of the acetyl groups from N-acetylhexosamine are included in the results. The amino-acids set free during the hydrolysis were estimated by the method of Van Slyke & Dillon [1938] as modified by Van Slyke, Dillon, MacFadyen & Hamilton [1941] and the results are expressed as 'carboxyl-N'. Glucosamine is not determined under these conditions. 'Hexosamine' was determined according to the method of Elson & Morgan [1933] and is expressed as glucosamine. N-acetylglucosamine was found to be converted almost completely to glucosamine by heating with N HCl at 100° for 2 hr. Reducing substances reach a maximum value after about 4 hr. heating, whereas the optical rotation, the 'hexosamine' and the amino-N reach maximum values between 6 and 12 hr. hydrolysis. The maximum figure for amino-acid is reached only after several hours' heating with 6N HCl.

The maximum figures for amino-acid N (2·2%) and for hexosamine-N (2·3%) are together approximately equal to the maximum amino-N value (4·5%). During the early stages of the hydrolysis, however, the total amino-N value (Van Slyke's method) is greater than the sum of the amino-acid and hexosamine figures. This is most probably due to the fact that the Van Slyke method assays the terminal NH_2 groups of polypeptides, whilst the amino-acid determination (ninhydrin method) is specific for free amino-acids only. There remains a considerable discrepancy between the maximum amino-N value (4·5%) and the total N of the preparation (5·7%). If, however, all the N (3·5%) not

Table 7. *The hydrolysis of A-substance by* HCl *at* 100°

Acid strength	Time of heating (hr.)	$[\alpha]_{5461}$ ($c=1\%$, $l=1$)	Reducing power (glucose =100)	Hexosamine* (%)	Nitrogen (%)			
					Total	α-Amino	Amino-acid	Hexosamine
N	0	+0·11°	0·5	0·0	5·72	0·19	0·0	0·0
	1	+0·35°	45	24·3	—	3·14	0·19	1·90
	6	+0·52°	50	26·9	—	4·02	0·46	2·05
	12	+0·51°	45	27·3	—	4·20	0·80	2·17
	24	+0·50°	—	26·6	—	4·29	1·32	2·24
6N	1	+0·44°	42	27·0	—	4·27	0·99	2·19
	6	+0·32°	43	24·0	—	4·55	2·17	1·88
	12	+0·24°	—	—	—	3·66	1·75	—

* Estimated by the colorimetric method.

The analytical figures obtained during the course of the hydrolysis of a typical preparation are given in Table 7. The figures largely confirm the results obtained earlier by Landsteiner & Harte [1940]. The reducing power was determined by Somogyi's modification [1926] of the Shaffer & Hartman method [1921] and the results are expressed in terms of glucose. The liberation of α-amino groups accounted for as hexosamine is assumed to be in the amino-acid component, it follows that the latter comprises about $3{\cdot}5\times6{\cdot}4$ or 22% of the total complex. The maximum figure obtained for reducing substances is 52%, calculated as glucose. Differentiation of the reducing substances into fermentable and non-fermentable components by the rapid yeast 'absorption' method of Harding & Selby [1931]

revealed that the fermentable sugar did not exceed 3–4 % of the maximum reducing substances at any stage of the hydrolysis. If the hexosamine present is considered as glucosamine, which has quantitatively the same reducing power as glucose under the conditions of estimation employed, then the hexosamine accounts for 27 % of the reduction. If the remaining reduction (25 %) is assumed to be due to galactose, then this sugar is present to the extent of 32 %, since its reducing power, relative to glucose, is only 72 %. Qualitative tests for the presence of individual amino-acids show that tyrosine and cysteine are absent or present in very small amounts.

DISCUSSION

The isolation of the specific A-polysaccharide substance from crude gastric mucin by methods that avoid the use of mineral acid or of alkali, and enable the isolation procedures to be carried out at normal temperatures, is described.

The methods give an excellent yield of a serologically active polysaccharide-amino-acid complex, which possesses in full the property shown by crude gastric mucin of inhibiting the isoagglutination of A-erythrocytes by natural anti-A (α)-agglutinin (human), retains completely the high viscosity of the original mucin, and is essentially homogeneous when examined at pH 4·0 or 8·0 in the Tiselius electrophoresis apparatus. The material can be considered as the blood group A-substance largely in its undegraded state and it is noteworthy that these two radically different methods for the isolation of the active substance yield apparently identical products.

The A-substance prepared by either method is similar in composition to that described by Landsteiner & Harte [1940]. There are, however, a number of important differences in the physical and serological properties of our preparations of A-substance and those described by other workers. Preparations of A-substance made by either of the methods described in this paper show not only high serological activity, as determined by the inhibition of isoagglutination, but also considerable viscosity, and the property of forming an elastic gel on the addition of borate buffer at pH 8·5. The latter property is readily lost and it has been found that even slightly degraded preparations of the A-substance fail to form an elastic gel on the addition of the borate buffer. Thus, the addition of this reagent to a solution of A-substance will reveal at once whether the material has been degraded during the course of its preparation. In addition to the rapid decrease in the viscosity of the A-substance that arises from heating solutions of the substance, there is a steady fall in the capacity of the preparation to inhibit the isoagglutination of A-cells by either natural human anti-A (α)-agglutinin or by the anti-A agglutinin of rabbit immune serum. The insensitive nature of the agglutination reaction as usually carried out, with progressively doubled dilutions of the A-substance, does not allow small changes in the capacity of an A-preparation to inhibit isoagglutination to be observed. For this reason it is not possible to detect a decrease in the power of a preparation to inhibit isoagglutination when the destruction is much less than 50 % of the original value. Nevertheless, it is apparent that heating at 100° or simple sterilization at 120° in neutral solution causes a loss in isoagglutination inhibiting activity, whereas almost complete destruction of this particular serological activity is brought about by heating with formamide or warming with dilute sodium carbonate. Although exposure to these conditions leads to drastic loss in viscosity and almost complete destruction of the power to inhibit isoagglutination, it is of interest to find that the resulting substance in each instance shows unchanged the property of inhibiting the haemolysis of sheep cells by anti-A rabbit immune serum. Indeed, there is evidence that heating with formamide may even increase this particular activity, as was originally observed by Landsteiner & Harte [1940]. These workers stated that the increase in anti-lytic activity is most probably due to a structural change, since 95 % of the starting material with about twice the potency could be recovered after this treatment. Jorpes & Norlin [1933; 1934] and Jorpes [1934] reported that the action of trypsin on the blood group A factor in human urine considerably decreased the anti-agglutinating activity but that the anti-lytic activity was not destroyed. They found that anti-agglutinating and anti-lytic factors could be separated by precipitating the anti-agglutinating factor from solution by tannin. Jorpes & Norlin consider that the anti-agglutinating factor is in this instance protein in nature, the anti-lytic substance being essentially polysaccharide. With material of high anti-lytic activity, we have not so far detected any chemical difference between preparations of the A-substance that show high or low activity in inhibiting isoagglutination and Landsteiner & Harte [1940] reported that they were unable to effect a separation of the A-substance into material showing different chemical and serological properties. Degradation of the native A-substance under the conditions devised by earlier workers for its isolation and purification almost certainly accounts for the low viscosity and much decreased power to inhibit isoagglutination, both of which are recorded in the literature for purified preparations, whereas the stability of the haemolysin-inhibiting property, when exposed to similar conditions, offers an explanation for the preservation of the intense anti-

haemolytic activity of these preparations. It must be emphasized that up to the present the preparations of A-substance, used by different workers, have been obtained almost exclusively from one source. This material, Wilson's gastric mucin (Type 1701-W), is the one most commonly used by bacteriologists for enhancing the virulence of bacterial cultures. We have examined one specimen of gastric mucin obtained in this country from Light and Co. and have found it to be very similar to the Wilson product. It is not known to what extent the A-factor in these commercial materials is degraded during their preparation. Our observations on the ease with which certain properties of the A-substance are destroyed suggest that further investigation of the properties of native gastric mucin should be undertaken with respect to its power to inhibit isoagglutination and A specific haemolysis and the relation of these activities to viscosity and power to increase the virulence of bacterial cultures. It is of interest that we have isolated from the fluid of pseudo-mucinous ovarian cysts a polysaccharide complex that is two or three times as active in inhibiting A-isoagglutination as the most active material isolated from hog gastric mucin. This material is, however, less viscous than hog gastric mucin A-substance.

It is not possible to compare accurately the activity of our preparations with those of other workers, because in many instances little or no indication is given of the strength of the anti-A (α)-agglutinin or of the anti-A rabbit haemolysin used in the tests. By replacing our test dose of anti-A (α)-serum by a quantity of serum only just sufficient to give complete agglutination of the A-cells used in the test, we found the amount of A-substance giving rise to a significant although incomplete inhibition of isoagglutination to be about 0·01 μg. Similarly, haemolysis-inhibition tests in which 1 M.H.D. only of the lytic agent was employed showed that as little as 0·001 μg. of the undegraded A-substance could be detected. Smaller absolute values which are, however, of doubtful value, can readily be obtained if the same pipette is used throughout to make the serial dilutions of the A-substance.

The question arises as to why the A-substance, which contains amino-acids as an integral part of the molecular complex and is superficially analogous to the O antigens of certain bacteria, is not itself antigenic. Closer examination shows, however, that the A-substance is not truly analogous to these bacterial antigens, which appear to be large polymolecular complexes built by the combination of polysaccharide and protein molecules through secondary valency bonds. Some of these biologically important aggregates can be dissociated into their component molecules with complete loss of the original specific antigenic property. The A-substance, however, is not separable into its component molecules by these procedures and it would appear, therefore, that the amino-acid components are in this instance more firmly linked, most probably by normal primary valency bonds, to the carbohydrate units of the polysaccharide molecules. Furthermore, it seems probable that the amino-acids are present either as single molecules or as short polypeptide chains and that the A-substance does not in fact contain an intact protein component. In support of this suggestion it has been shown that the A-substance can be converted into a full antigen that gives rise to specific A immune-body, by combination with a suitable protein molecule. The conditions employed to synthesize this functional polymolecular aggregate suggest that the component molecules—A-substance and conjugated protein—are held together by secondary valency bonds only. An additional factor, however, that may well be responsible for the non-antigenicity of the A-substance, is its lack of tyrosine. The absence of this amino-acid has generally been considered to render a protein devoid of antigenic properties, although some evidence against this view has recently been given.

The preparation of the purified A-substance in large amounts has permitted an investigation into the nature of the constituent in commercial gastric mucin that is responsible for its 'virulence enhancing' power, a property which bacteriologists and immunologists have put to much practical use during the last few years. The undegraded A-substance appears to possess 'virulence enhancing' properties, whereas all other substances in gastric mucin, including the phenol-soluble protein component, are inactive in this respect. Any treatment, however, such as sterilization by heat, which destroys the viscosity of the A-substance and reduces the capacity of the preparation to inhibit isoagglutination (even when the power of the resulting material to inhibit the haemolysis of sheep cells by anti-A serum is completely retained) leads to destruction of the 'virulence enhancing' property.

SUMMARY

1. Two methods are described for the isolation from crude gastric mucin of a polysaccharide-amino-acid complex showing intense blood group A specificity.

2. The undegraded A-substance, which is essentially homogeneous, gives rise to slightly opalescent 1 % aqueous solutions that show considerable viscosity. The substance inhibits, in extremely small amounts, specific isoagglutination and haemolysis of sheep cells by specific anti-A rabbit immune serum.

3. The native A-substance is readily degraded by heat treatment and gives rise to a substance which shows a lower viscosity in aqueous solution and which possesses a reduced power to inhibit isoagglutination. The partially degraded A-substance, however, shows a slightly increased activity in inhibiting A specific haemolysis.

4. The relationship of the purified undegraded A-substance to the 'virulence enhancing' agent in crude gastric mucin is discussed.

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ADDENDUM

Electrophoretic Examination of A-substance from Gastric Mucin

By R. A. KEKWICK

Three preparations of A-substance from gastric mucin (210, 240, 321) were examined in the Tiselius U-tube at 0° with the diagonal schlieren system for optical observation. Samples of the dried preparations were dissolved in buffers to give 2% solutions and dialyzed in cellophane membranes at 2° against several changes of the same buffer. Buffers used were acetate, pH 4·0, $\mu = 0{\cdot}1$, phosphate, pH 8·0, $\mu = 0{\cdot}1$ and phosphate-NaCl buffer, pH 8·0 (phosphate $\mu = 0{\cdot}1$ + NaCl $\mu = 0{\cdot}15$). In all these buffers very viscous solutions were obtained, which gave a considerable light scattering.

In all samples, both at pH 8·0 and pH 4·0, a single component accounted for 90% of the total refraction, the remaining 10% being associated with a somewhat ill-defined contaminant.

In pH 8·0 phosphate buffer $\mu = 0{\cdot}1$, the migration was anodic, $\mu = 0{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$, and in pH 4·0 acetate buffer $\mu = 0{\cdot}1$ a very slight cathodic migration occurred. Under each of these conditions the descending boundary remained sharper than the ascending, the reverse of what is usually observed, and this presumably was due to the high viscosity of the solution.

Studies on the 'Fermentation' of Ceylon Tea

4. ESTIMATION OF THE OXIDIZING ENZYME ACTIVITY

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In our previous studies [Lamb & Sreerangachar, 1940*a*, *b*] the oxidizing activity of the tea-leaf enzyme was measured by following the changes in the tannin content of an enzyme-substrate reaction mixture by the iodine titration method. In stressing the limitations of this method it was stated that it did not afford any indication of complex changes such as condensation, and there existed a possibility that the results were subject to an error due to these causes.

The oxidizing enzyme in tea leaf is a polyphenol oxidase and ascorbic acid is only indirectly oxidized in the presence of a suitable polyphenol. The function of the polyphenol in this reaction is that of an oxygen carrier, and its permanent oxidation becomes noticeable only after the complete oxidation of all ascorbic acid. The *o*-quinones are continually removed from the sphere of reaction and therefore their condensation cannot occur as long as there is an excess of ascorbic acid in the system. The ascorbic acid reaction thus provides the basis of a more reliable method for measuring enzyme activity in the tea-leaf enzyme preparation. Ezell & Gerhardt [1940] have likewise employed ascorbic acid as a substrate in the determination of oxidase activity in fruits and vegetables.

EXPERIMENTAL

Enzyme preparation and properties. The enzyme preparation employed in this investigation was obtained by grinding tea leaf with sand under acetone, washing until free of all colouring constituents and rapidly drying under vacuum. This acetone-prepared leaf powder was all but polyphenol-free and exerted only a very weak oxidizing action on pure *l*-ascorbic acid. Gradual removal of the associated polyphenols from this tissue powder rendered the enzyme progressively inactive towards ascorbic acid. Thus on extraction with water and exhaustive washing, a large fraction of the residual tannin was removed and the ascorbic acid oxidation activity fell markedly. Aeration for 2–3 hr. of a suspension in water of the extracted enzyme powder did not remove any further quantities of polyphenol; grinding the extracted powder with sand and water, filtering and washing resulted in the material still giving a positive ferric chloride reaction for tannin substrates. However, when the ground mass was treated with an excess of a dilute solution of ascorbic acid it was found possible to extract completely the last traces of polyphenol. The ascorbic acid oxidation activity of this final preparation is almost negligible. Table 1 gives results showing both the polyphenol oxidase activity on a prepared tea-tannin substrate solution measured iodometrically and the ascorbic acid oxidation activity estimated by the use of the 2:6-dichlorophenol-indophenol method at the above different stages of extraction. The reactions were all carried out in presence of McIlvaine's buffer at pH 5·4.

Table 1. *Effect of polyphenol removal on enzyme activity*

Extraction stage	Activity/0·1 g. enzyme powder: Polyphenol oxidase activity (4 hr.) (ml. N/20 $Na_2S_2O_3$)	Activity/0·1 g. enzyme powder: Ascorbic acid oxidation (5 min.) (mg. ascorbic acid)
1. Unextracted powder	10·5	0·29
2. After extraction with McIlvaine's buffer at pH 7·0 and complete washing	12·8	0·09
3. After aeration	12·0	0·07
4. After grinding with sand and water	—	0·06
5. After treatment with ascorbic acid	18·4	0·002

These results indicate that the enzyme in question is not ascorbic acid oxidase but that it is polyphenol oxidase, of which the activity increases on partial purification. Since the complete removal of all tissue-bound polyphenols renders the enzyme practically inactive towards ascorbic acid there can be no reasonable doubt that the ascorbic acid oxidation activity of the unextracted powder was due to an indirect action of a polyphenol-polyphenol oxidase system.

The ascorbic acid oxidation activity exhibited by the enzyme in the presence of polyphenol was, however, found to vary according to the amount of polyphenol present. It became, therefore, necessary to find out the optimum quantity of polyphenol that would give the maximum enzyme activity. Two polyphenols, catechol and amorphous tea tannin, were so studied and Table 2 shows the effect of their addition on ascorbic acid oxidation by the enzyme. The reaction mixture consisted of 5 ml. 0·1% ascorbic acid solution, 4 ml. distilled water, 1 ml. catechol or tea-tannin solution containing various amounts of polyphenol, and 20 mg. acetone-prepared enzyme powder. The mixture was aerated for 1 hr., after which it was filtered and 5 ml. titrated with a standard solution of 2:6-dichlorophenol-indophenol. Neither 100 mg. catechol nor 20 mg. tea tannin, the maximum amounts of polyphenols employed in this experiment, gave any blank 2:6-dye titre when dissolved in 5 ml. A control experiment in which the polyphenol

solution was replaced by 20% solution of trichloroacetic acid was also run at the same time, and the enzyme activity obtained by the difference between the control and experimental values.

Table 2. *Effect of addition of polyphenols on ascorbic acid oxidation activity*

	Catechol			Tea tannin	
	Enzyme activity (mg. ascorbic acid)			Enzyme activity (mg. ascorbic acid)	
mg. added	(*a*)*	(*b*)*	mg. added	(*a*)*	(*b*)*
0	0·27	0·98	0	0·80	0·98
0·5	0·82	—	0·5	1·16	—
1·0	0·96	—	1·0	1·20	—
2·0	0·92	—	2·0	1·59	—
3·0	0·98	—	4·0	1·63	—
4·0	1·06	—	5·0	—	3·77
5·0	1·33	—	10·0	—	4·06
10·0	1·53	—	20·0	—	5·02
15·0	1·94	—	40·0	—	4·53
20·0	2·10	—	—	—	—
25·0	2·22	2·90	—	—	—
50·0	—	2·04	—	—	—
100·0	—	1·22	—	—	—

* (*a*) and (*b*) represent two separate enzyme preparations.

The results (Table 2) show that in a total volume (10 ml.) of the reaction mixture either 25 mg. catechol or 20 mg. tea tannin gave optimum values for ascorbic acid oxidation activity, which is higher in the case of tea tannin than catechol. But as the preparation of tea tannin in pure state is tedious and its identity still uncertain it was thought desirable to employ only the more definite compound, catechol. Instead of using catechol and ascorbic acid solutions separately it is convenient to prepare a mixed substrate solution containing 0·1% ascorbic acid and 0·5% catechol. 5 ml. of this solution, containing 5 mg. ascorbic acid and 25 mg. catechol, are usually employed in each reaction.

Optimum pH *and temperature.* The optimum conditions for ascorbic acid oxidation by the tea-leaf enzyme system are shown in Table 3. McIlvaine's buffer at various pH values was employed and the reactions for determination of optimum temperature were carried out in a thermostat at pH 5·4. There was slight autoxidation of ascorbic acid at pH > 6·0 and the values were corrected for such autoxidation errors.

Table 3. *Optimum* pH *and temperature*

	Enzyme activity (mg. ascorbic acid)			Enzyme activity (mg. ascorbic acid)	
pH	Mixed enzyme	Insoluble enzyme	Temp. (°C.)	Mixed enzyme	Insoluble enzyme
4·0	2·34	2·00	16	1·14	1·22
4·6	2·48	1·80	21	1·59	1·47
5·0	2·85	—	27	1·79	1·92
5·4	2·73	2·53	32	1·26	1·71
6·0	1·82	1·84	38	1·06	1·29
7·0	1·49	1·13			
8·0	1·36	1·18			

The mixed and insoluble enzymes were derived from different samples of leaf. The optimum pH for the mixed and the insoluble enzymes is 5·0 and 5·4 respectively, while both enzymes have the same optimum temperature, 27°.

Relation between soluble, insoluble and mixed enzymes. In earlier investigations it was shown that the activity of the unextracted powder was not equal to but always less than the sum of the activities of the extract and the residue. When this question was reinvestigated by the ascorbic acid method, it was observed that the activity of the unextracted powder consisted of those due to the soluble and insoluble enzymes. To a mixture of 5 ml. of ascorbic acid-catechol substrate and 5 ml. McIlvaine's buffer at pH 5·4 the enzyme was added in the following forms: (1) unextracted powder (20 mg.), (2) insoluble enzyme from 20 mg. unextracted powder, and (3) enzyme extract (2 ml.) derived from 20 mg. unextracted powder. The reactions were carried out separately. At the end of 1 hr., the enzyme activities were estimated by titration of 5 ml. filtrates in the case of solid enzymes and 6 ml. in the case of the enzyme extract. The results are shown in Table 4.

Table 4. *Activities of soluble, insoluble and mixed enzymes*

	mg. ascorbic acid oxidized		
	I	II	III
Soluble enzyme	0·82	0·41	0·28
Insoluble enzyme	3·56	1·41	0·90
Mixed enzyme	4·32	1·80	1·15

It can be concluded that the activity of the mixed enzyme represents the total of the soluble and insoluble enzyme activities, and therefore the acetone-prepared enzyme can be employed in any comparative study of the enzyme contents of tea-leaf samples. Condensation is eliminated in the present method, and it is possible that the explanation of the discrepancy between these results and the previous ones is in some way connected with this condensation phenomenon.

Effect of concentration of enzyme. That the activity is proportional to the amount of the reacting enzyme is shown (Table 5) by an experiment in which the quantities of the enzyme were varied, keeping the other conditions the same. The period of reaction was, however, reduced to 30 min. in order to compare the activities while the reaction is proceeding briskly.

Table 5. *Effect of enzyme concentration*

Quantity of enzyme powder (mg.)	Activity (mg. ascorbic acid)
5	0·3
10	0·58
20	1·17
40	2·45
60	3·57

The ascorbic acid method can therefore be employed in tea-fermentation studies, especially in cases where it is required to assess or compare the enzyme activities. Such an application of the method has already been made in the study of enzyme activity in individual bushes.

Description of the proposed method and its advantages

Acetone-prepared enzyme powder (20 mg.) is weighed into a 50 ml. test-tube containing 5 ml. McIlvaine's buffer at pH 5·0 and 5 ml. catechol-

ascorbic acid substrate solution. The mixture is aerated for 1 hr., and 5 ml. filtrate are titrated in presence of 1 ml. 20% trichloroacetic acid with 2:6-dichlorophenol-indophenol solution standardized according to the method of Menaker & Guerrant [1939] for its ascorbic acid equivalent.

A control with the same quantities of enzyme and substrate but with 5 ml. 10% trichloroacetic acid solution instead of buffer is also run at the same time. The difference between the control value, which provides the initial amount of ascorbic acid, and the experimental result gives the amount of ascorbic acid oxidized during the reaction or, in other words, the enzyme activity. It has been established by a separate control that there are no autoxidation errors in the reaction at pH 5·0. No difficulty has been encountered in the dye titration of ascorbic acid due to the normal colour of either the enzyme extracts from tea leaf or its juice.

The iodometric method of measuring enzyme activity depends upon estimations of tannin by iodine titration, the limitations of which have been already stressed. The present method, on the other hand, has several advantages: (1) Both the chemicals used in substrate solution are of well-defined constitution and can be obtained in a high degree of purity. (2) The method is fairly rapid and does not involve the necessity of observing a standard time of reaction during titrations. (3) Greater accuracy is obtained not only in titrations but also from the fact that condensation errors are eliminated. (4) In iodometric titrations it was either necessary to obtain a completely tannin-free enzyme preparation or a control had to be run to determine the amount of buffer-extractable polyphenols. In the ascorbic acid method this is obviously unnecessary, as polyphenols are in fact added to the reaction mixture.

DISCUSSION

Condensation changes which follow the accumulation of *o*-quinones in a reaction mixture can take place between two or more molecules of *o*-quinones themselves or between one molecule of *o*-quinone and one or more molecules of unoxidized polyphenol. Such condensed products are oxidized by iodine relatively more slowly than the original polyphenols, and this fact is reflected in an apparent fall in their iodine titre. Some indication of this is afforded by our results on catechol oxidation by oxidase and peroxidase [Lamb & Sreerangachar, 1940*b*]. Oxidase activity produced very little change in iodine titre, whereas peroxidase produced a marked fall in titre, and it appeared that peroxidase activity resulted in the formation of more condensation products than does oxidase activity.

The criticism of Shaw's iodometric method for the volumetric estimation of tea tannin by Barua & Roberts [1940] is based on this question of condensation of tannin bodies after oxidation and the errors that may be introduced thereby. Reference to Table 3 shows that the conditions of optimum pH and temperature for the action of the tea-insoluble enzyme are almost the same when determined by the iodometric method previously used and by the present ascorbic acid method, which is independent of any interference due to condensation. Our previous conclusions, which were mostly based on the iodometric method, may not then be substantially affected by errors due to condensation.

An explanation of this agreement may perhaps be suggested. As condensation is a chemical reaction, it is reasonable to assume that the rate and amount of condensation of oxidized tannins will bear a definite relation to the amount of oxidized tannins present, which in its turn will be related to the activity of the enzyme. The fall in iodine titre due to the condensation factor would, therefore, vary in proportion to the enzyme activity itself, so that the method can still be employed in any comparative study of enzyme activities.

Further investigation is needed to amplify our present insufficient knowledge about this condensation process of oxidized tea tannin bodies. For example, it is not known whether or not the condensation phenomena are attended by any further oxygen uptake or even by simple dilatometric changes, in which case the manometric results on the fermentation of tea may be regarded as subject to an error.

SUMMARY

1. A new method of estimating oxidase activity in tea, by the use of ascorbic acid as substrate, has been described. The method is simple, accurate and avoids errors due to condensation of oxidized polyphenols, which was the chief drawback of the iodine titration method.

2. The values for the optimum pH and temperature of the insoluble enzyme are confirmed by this new method.

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Studies on the 'Fermentation' of Ceylon Tea

5. COMPARATIVE RATES OF OXIDATION OF DIFFERENT POLYPHENOLIC SUBSTRATES BY TEA-OXIDIZING ENZYMES

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The oxidizing-enzyme preparation employed in previous work was obtained from tea by grinding fresh leaf under acetone until the residue was almost completely free of all colouring constituents of the leaf.

Among the differences that exist between the *in vitro* action of this acetone-prepared tea enzyme and the *in vivo* reaction in the minced leaf are the rates of oxidation of different polyphenolic substrates. Thus, acetone-prepared enzyme oxidizes catechol about two or three times as rapidly as it does tea tannin [Lamb & Sreerangachar, 1940], whereas in the fermenting leaf-mince to which the polyphenolic substrates are introduced after completion of its initial fermentation, tea tannin is oxidized at about three times the rate of catechol oxidation [Harrison & Roberts, 1939].

There is obviously one main difference between the conditions that exist in the minced-leaf system prior to the addition of the polyphenolic substrates, and those obtaining in the acetone-prepared enzyme reaction system. In the former, the leaf polyphenols are, as a result of initial reactions, in various stages of oxidation and condensation, whereas in the latter such oxidized products of tea tannin are absent at the commencement of the reaction. The object of the present investigation is to find out the exact influence of these oxidized polyphenols on subsequent oxidation reactions, and to arrive at a clear distinction between the direct reactions of the enzyme and the possible indirect action of other substances present.

EXPERIMENTAL

Methods. The rates of oxidation of different polyphenols by tea enzymes, as shown by their oxygen uptake, were measured at 25° with Barcroft's apparatus [cf. Sreerangachar, 1941]. Observations included reactions in the fresh-leaf mince and also *in vitro* reactions of prepared enzyme and substrate. In the latter type of reaction, the enzyme powder was initially kept floating on the substrate solution in small 'pliofilm' boats which could be upset whenever desired by shaking the apparatus, thus bringing the enzyme and substrate into contact with each other. Any complication due to respiration changes was avoided by not introducing KOH into the central tube. Such a contingency arose only when fresh-leaf mince was used; for in the case of *in vitro* reaction mixtures the system was entirely free from living tissues. The polyphenolic substrate solutions employed were all of M/10 concentration. The amorphous tea tannin was prepared by the Tocklai modification of Shaw's method [1935], and all gallic acid was removed from it by extraction with dry ether.

Substrate specificity. Attention was first directed towards finding out the relative rates of oxidation

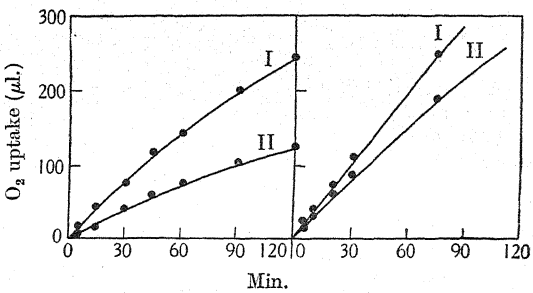

Fig. 1. Oxidation by acetone-prepared tea enzyme. I, catechol; II, tea tannin.

of catechol and tea tannin by the use of the same enzyme preparation. Acetone-prepared enzyme powder (50 mg.) was allowed to react on 2 ml. substrate solution to which 2 ml. McIlvaine's buffer solution at pH 5·0 were added. Fig. 1 represents the course of oxygen uptake by two such systems, and it will be seen that the rate of oxidation of catechol is higher than that of tea tannin, although the difference is not as great as that obtained previously with different enzyme preparations. This has been confirmed in several experiments.

The rate of oxidation of a few other substrates available here, namely, pyrogallol, ascorbic acid and quinol, were then studied in comparison with catechol and tea tannin (Fig. 2). The enzyme oxidizes catechol, tea tannin and pyrogallol, members of a class of substrates to which the enzyme is specific, more rapidly than it does ascorbic acid

and quinol, which do not belong to this specific class. The slight oxidizing action observed on ascorbic acid and quinol is due to the indirect influence of minute quantities of original polyphenols in the enzyme powder which resist complete extraction with acetone.

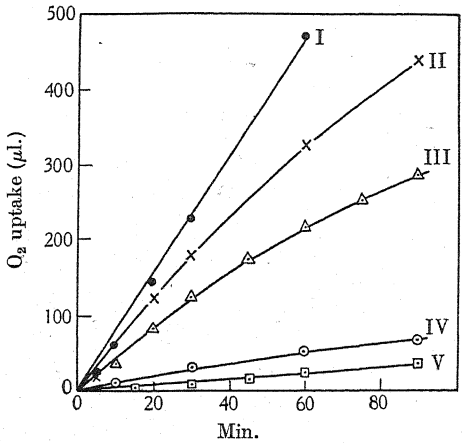

Fig. 2. Oxidation of polyphenols by acetone-prepared enzyme. I, catechol; II, tea tannin; III, pyrogallol; IV, ascorbic acid; V, quinol.

The oxygen uptake of the leaf-mince suspension came almost to a standstill after about 90–120 min. and the relative rates of oxidation of polyphenolic substrates were then measured by introducing them at this stage to the leaf-mince system. It was observed that tea tannin was oxidized more rapidly than catechol as shown in Fig. 3, I. Harrison & Roberts [1939] in Assam also found that tea tannin is oxidized about three times as rapidly as catechol with minced leaf.

Influence of oxidation products. A study of these results suggested that the tea polyphenols existing in different stages of oxidation and condensation as a result of the initial fermentation reaction in the leaf mince might probably be the chief cause of the difference between the results with acetone-prepared enzyme and those obtained with leaf mince. The interference of the oxidized products of tea polyphenols with the oxidation rates of polyphenols subsequently added might have been so pronounced as even to reverse the relative rates of oxidation of tea tannin and catechol. In order to test this hypothesis, fresh-leaf mince was repeatedly washed with water after its initial reaction, to remove the accumulated oxidation products, and the residue allowed to react on catechol and tea tannin. It was then found that the oxidation of tea tannin not only ceased to be faster than that of catechol but its rate was either the same or even lower than that of catechol as shown in Fig. 3, II.

The use of acetone, to remove the oxidation products from the leaf mince after the initial reaction, yields still better results, due to a greater extraction of these materials, though perhaps not so completely as in the preparation of enzyme from

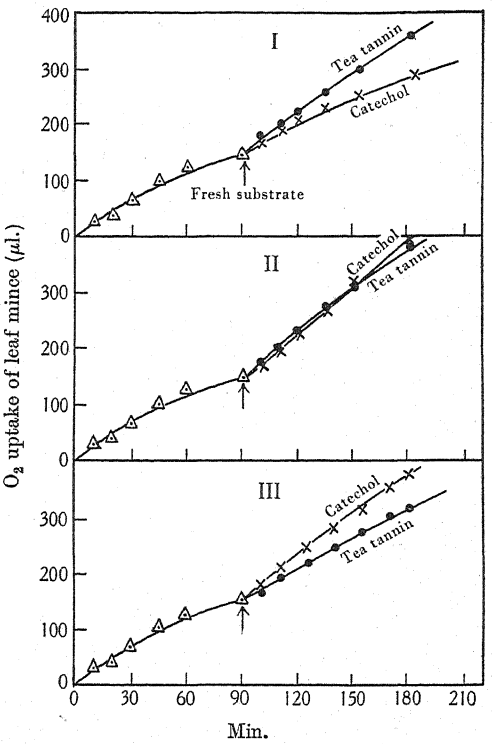

Fig. 3. O_2 uptake of leaf-mince suspensions with added substrates. I, reactions in presence of tannin oxidation products; II, ditto after removal of these by washing the tissues; III, ditto after removal by acetone extraction.

fresh leaf. Comparison with the results of water extraction reveals that acetone treatment does not appreciably inhibit the enzyme with respect to its rate of polyphenol oxidation. The rates of oxidation of catechol and tea tannin by the acetone-extracted residue, from leaf mince which had undergone the initial fermentation reaction, are in the same order as those observed in the case of acetone-prepared enzyme, although in the former case the difference between the oxidation rates of catechol and theotannin is not as great as in the latter. Fig. 3, III shows that catechol is being oxidized slightly more rapidly than tea tannin by the acetone-extracted residue of leaf mince which had previously been fermented.

Further confirmation of the influence of the tea-tannin oxidation products on subsequent oxidations of catechol and of tea tannin was obtained when these products, from another experiment, were

added to the reaction mixtures of the acetone-prepared enzyme on the one hand and catechol and tea tannin on the other. The relative rates of reaction at once changed, tea tannin being then oxidized more rapidly than catechol.

When this evidence concerning the influence of tea-tannin oxidation products on the subsequent oxidations of catechol and tea tannin had been gathered it was of interest to find out whether such behaviour was peculiar only to the tea-tannin oxidation products or whether it was generally true of the oxidized products of other polyphenols as well. The general scheme of the experiment was that the oxidized products of three phenolic bodies, catechol, quinol and tea tannin, should be added at the commencement of the reaction to systems each containing one of these polyphenols and the acetone-prepared enzyme. Comparisons of oxygen uptake results of two otherwise corresponding systems, with and without the addition of oxidation products, would reveal the effect, if any, of such additions. The oxidized products of catechol and tea tannin were obtained by oxidizing M/20 solutions of these polyphenols by acetone-prepared enzyme. It was not necessary to prepare experimentally the oxidized product of quinol, as pure *p*-quinone was available. A M/20 solution of *p*-quinone was used. Solutions (2 ml.) of these three oxidized polyphenols were added to each of the polyphenol-enzyme mixtures and the oxygen uptake measured by manometric methods. Fig. 4 represents the results obtained. The results shown in dotted lines (Fig. 4, A, D) were obtained with a separate sample of enzyme, as the sample used in the case of all other reactions became exhausted, a circumstance which cannot, however, affect the conclusions. These results are summarized in Table 1.

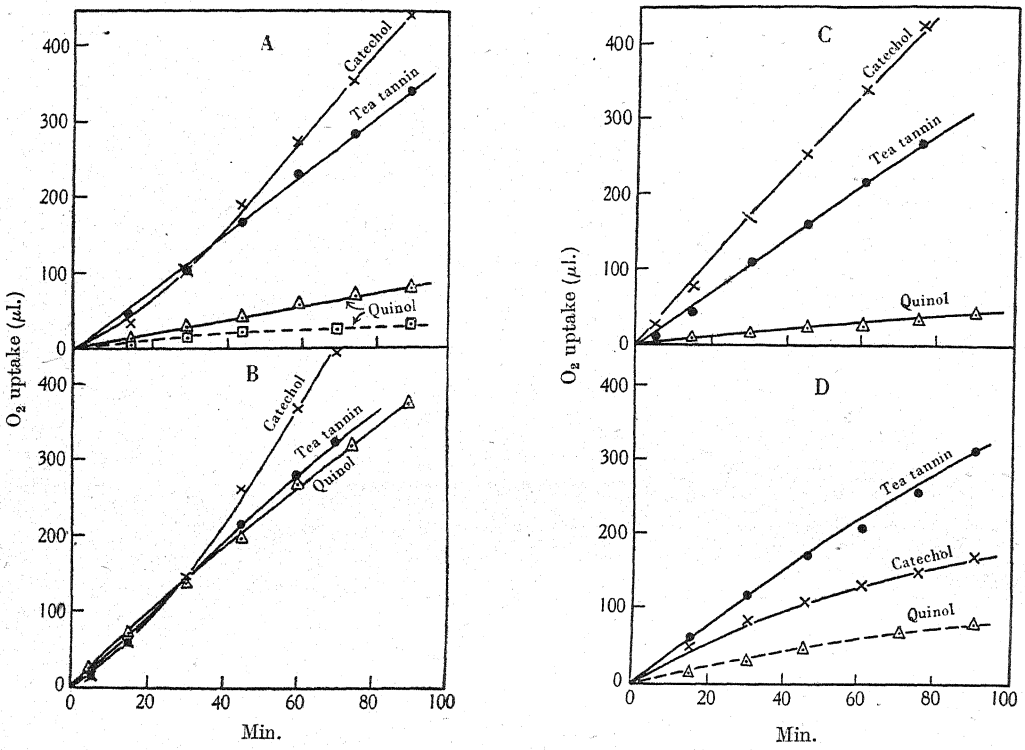

Fig. 4. A, oxidation of polyphenols by acetone-prepared enzyme from tea; B, reactions with added catechol oxidation products; C, ditto with added *p*-quinone; D, ditto with added tea-tannin oxidation products. Dotted lines in A and D represent reactions with a different enzyme.

Table 1. *Oxygen uptake during various polyphenol oxidations*

Product added	Polyphenol oxidation, μl. O_2/hr./mg. enzyme: Catechol	Tea tannin	Quinol	Quinol
Nil	5·5	4·6	1·2	0·4*
Catechol oxidation products	7·4	5·6	5·3	—
p-Quinone	6·8	4·4	0·6	—
Tea tannin oxidation products	2·6	4·3	—	1·2*

* A different sample of enzyme.

Catechol oxidation products accelerate oxidations of catechol and tea tannin almost to the same extent, while quinol oxidation is increased four to five times. *p*-Quinone accelerates catechol oxidation and does not much affect tea-tannin oxidation; but it markedly inhibits quinol oxidation. Tea-tannin oxidation products increase the rate of quinol oxidation nearly three times but inhibit catechol oxidation to less than half its original activity. Oxidation of tea tannin also suffers a slight inhibition. These results point to the general conclusion that the oxidation of polyphenols is markedly affected by the presence, at the beginning of the reaction, of certain polyphenol oxidation products. There appears to be, however, no correspondence between the polyphenol reaction affected and the polyphenol-oxidation products which influence the reaction. In particular, the reversal of the rates of oxidation of catechol and tea tannin in presence of oxidation products of the latter appears to be due to a marked depression of catechol oxidation, but not to an acceleration of the tea-tannin oxidation.

Corroboration of this point is noticeable in the results of fresh leaf-mince reactions and those with washed tissues. The oxygen uptakes during tea-tannin oxidation of 200 mg. leaf mince in 1 hr. (Fig. 3) are 135 and 150 μl. for unwashed and washed tissues respectively, while the corresponding figures for catechol oxidation are 98 and 163 μl. for the same unwashed and washed tissues. The marked inhibition of catechol oxidation by tea-tannin oxidation products is thus indicated.

When tea tannin is oxidized either in the fermenting leaf or *in vitro* by the prepared enzyme, the product consists of the oxidized tannins in a large range of condensations. In the present state of our knowledge about the chemistry of tea it is not possible to distinguish between the simple tea tannin-*o*-quinone and its various condensation products, or to ascertain their relative amounts in a solution of oxidized tea tannin. For this reason, the effect of tea-tannin oxidation products on subsequent polyphenol oxidations cannot be attributed singly either to the one or the other of these constituents. The following experiment, however, affords some evidence on this point. Condensation in a solution of oxidized tannin can be increased by rendering the solution alkaline, and it was desired to find out whether such an increase in the condensation products would materially affect the results. Five ml. of an oxidized tea tannin solution (M/20) were made alkaline to pH 9·5–10·0, boiled and cooled, and the pH readjusted to 5·0. This solution and 5 ml. of original solution were both made up to 10 ml., and 2 ml. of each were added to the oxidation-reaction systems of catechol and tea tannin.

It can be concluded from the results (Fig. 5) that increasing the condensation products has no significant effect on the rate of tannin oxidation, while it slightly enhances the inhibition of catechol oxidation. Therefore, the inhibition effect observed in catechol oxidation by addition of tannin-oxidation products is probably to be associated more with the

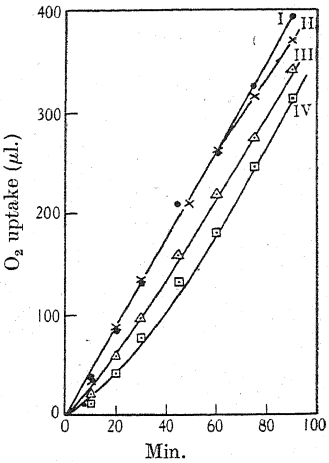

Fig. 5. Oxidations of catechol and tea tannin in presence of tea-tannin oxidation products. I, tea-tannin oxidation, lower condensed products; II, ditto, higher condensed products; III, catechol oxidation, lower condensed products; IV, ditto, higher condensed products.

higher condensation products of tannin-*o*-quinone than with the simpler tannin-*o*-quinone itself. The difference between the rates of catechol oxidation with the two concentrations of condensed products is, however, small, but it may probably be due to the fact that the original solution of oxidized tea tannin which was standing for 24 hr. already contained an effective amount of condensed tannin-oxidation products, which might have been increased only slightly on the solution being rendered alkaline.

Reaction in a mixture of substrates. When a single enzyme oxidizes two substrates, the rate of oxidation of their mixture generally falls between the two individual values owing to the competition of substrates for the enzyme. An investigation of the action of the acetone-prepared enzyme on a mixed substrate of catechol and tea tannin showed (Fig. 6) that the initial rate of oxidation of the mixture was intermediate between the rates of individual oxidations of catechol and tea tannin. This is in accordance with the above general rule and indicates that a single enzyme is concerned in the reaction. Towards the end of the reaction, however, the accumulation of the oxidation products begins to exert its influence and tends to depress the rate of reaction.

Addition of catechol to the fermenting leaf leads to conditions in which the same tea enzyme is acting simultaneously both on catechol and tea tannin. The rate of oxidation would then be intermediate between the oxidation rates of the two individual substrates. Harrison & Roberts [1939], however, found in a similar experiment that the rate of oxidation of the mixture was higher than that of the individual substrates. In arriving at this conclusion they made use of their previous result that catechol is oxidized at about one-third the rate of tea tannin, a result which was obtained without taking into consideration the presence and, therefore, the influence, of tannin-oxidation products. But, in view of the facts shown in the preceding section, the validity of this conclusion is open to doubt. When the individual rates of oxidation of catechol and tea tannin are investigated under conditions free from any extraneous interference, it is possible that the rate of oxidation of the mixture of substrates by the fermenting leaf system is actually intermediate between the two individual values.

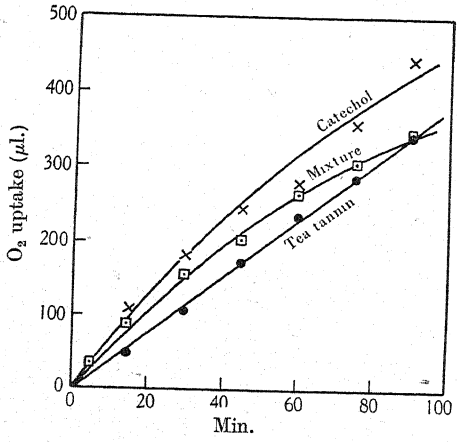

Fig. 6. Oxidation of catechol, tea tannin and their mixture by acetone-prepared enzyme.

DISCUSSION

Roberts [1941] has suggested that the difference in the rates of oxidation of catechol and tea tannin observed at Tocklai and St Coombs is due to the presence of two different enzymes in the materials derived from the two different sources. In the literature there is, no doubt, some evidence for the presence of more than one oxidase system in the same plant tissue [Boswell & Whiting, 1938; Marsh & Goddard, 1939]. But in this instance such a suggestion seems unnecessary, since this particular disagreement between St Coombs and Tocklai is due more to a difference in technique than to the nature of the materials. The reaction in the minced leaf as employed by Roberts is complicated by the accumulated tannin-oxidation products, which exert some influence on the course of the subsequent reactions.

SUMMARY

1. The relative rates of oxidation of catechol, tea tannin, pyrogallol, quinol and ascorbic acid by the tea enzyme prepared with acetone have been studied by manometric methods.

2. The discrepancy in the comparative rates of oxidation of catechol and tea tannin between our results and those of Assam workers has been shown to be due to an inhibitory effect, under the conditions of Assam experiments, of the condensation products of oxidized tea tannin on the oxidation of catechol. In view of this, the supposition of two different enzymes in Assam and Ceylon seems unnecessary.

3. It is shown that the presence of oxidized products of polyphenols at the commencement of a subsequent polyphenol oxidation reaction affects, in general, the course of such reaction.

4. The action of the enzyme prepared with acetone in a mixture of catechol and tea-tannin substrates has yielded results consistent with the view that both these substrates are oxidized by a common enzyme, namely, the tea polyphenol oxidase.

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Studies on the 'Fermentation' of Ceylon Tea

6. THE NATURE OF THE TEA-OXIDASE SYSTEM

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Recent investigations on tea fermentation, both in Ceylon and Assam, have led to distinct differences of opinion regarding the nature of the oxidase system that is responsible for fermentation. After modifying his earlier views on this question at least twice, Roberts [1939; 1940] has now identified the tea-oxidizing enzyme with cytochrome oxidase. In previous studies from these laboratories [Lamb & Sreerangachar, 1940], not only have objections to the cytochrome theory of tea fermentation been presented, but also definite evidence for the view that tea oxidase is a polyphenol oxidase with an established specificity for *o*-dihydric phenols.

Despite this, the considerable body of indirect evidence that Roberts adduced in favour of his theory made it appear for the moment a reasonable assumption, but further work has shown that alternative explanations could be found for most of his material observations. Perhaps the most serious objection to the cytochrome theory of tea fermentation is that so far there is no direct evidence for the presence in tea leaf of cytochrome and cytochrome oxidase, which seems quite essential for the acceptance of the theory. The present investigation was, therefore, undertaken with a view to obtaining some definite information which would serve either to establish or confute the presence of these components in tea.

Spectroscopically, cytochrome is easily detected in its reduced state by its characteristic absorption spectrum. Since chlorophyll may interfere in the observations on the leaf it may be extracted by solvents such as acetone. Any possible inactivation of the oxidase in the process does not affect either the content or the character of cytochrome. Following this procedure, Yakushiji [1935] has reported seeing the α-bands of cytochrome *b* and *c* in acetone-extracted spinach leaves. Choosing the basal portion of the stem, probably with a view to obviating the chlorophyll interference, Roberts [1941], with Miss K. Bhagvat, claims to have demonstrated the presence of cytochrome in the tea plant. They appear to have observed an absorption band corresponding to the '*b*' band of cytochrome in the green region of the spectrum. It is not clear whether it is the component *b* or the spectral band β that they have observed, and it is noteworthy that other bands of either cytochrome *b* or *c* (it cannot be component *a* as its β band is unknown) are not observed.

Spectroscopy can also be used in detecting the presence of cytochrome oxidase. On oxidation by the enzyme the sharp absorption bands of reduced cytochrome give place to the diffuse bands of oxidized cytochrome which are hardly perceptible in the micro-spectrophotometer. Disappearance of reduced cytochrome bands would therefore indicate the action of the cytochrome oxidase.

Before proceeding to describe the experimental evidence in the present investigation, a brief reference may be made to those points of dissension between workers here and in Assam not previously discussed by us.

Methods of investigation. In earlier work we have relied mostly on the titrimetric methods in the estimation of the tea-oxidase activity, and we have recognized the limitations of the method. Roberts [1941] has criticized this method on the ground that the iodine titre is affected by condensation of *o*-quinones produced, but has admitted that these errors are likely to be very small.

However, an alternative method of estimating enzyme activity, using ascorbic acid as substrate as described in Part 4 of this series, has yielded identical results as regards the optimum pH and temperature of the enzyme reaction. Therefore condensation errors in the iodine titration method could only be very small, and in a comparative experiment the conclusions are practically unaffected by these errors. It is interesting to note in this connexion that Roberts [1941] has himself obtained results of optimum pH (5·4) and temperature (29°) which are very close to those found by us. Such correspondence detracts very considerably from the relevance of his criticisms of the accuracy of our method. From the point of view of our present contention about the specific nature of the oxidase, the quantitative accuracy of the method is relatively less important. It is sufficient that the direct oxidase activity of the enzyme on polyphenols has been demonstrated beyond doubt.

The study of simpler component reactions affords an easy approach to the understanding of the most complicated biological processes *in vivo*. Preparation of purified enzyme and a study of its reaction *in vitro* on pure chemical substances is therefore a fruitful line of investigation [e.g. Hussein & Cruess, 1940], and we have always followed this course in our study of the mechanism of tea fermentation and the substrate specificity of the enzyme.

Substrate specificity. The importance of this property of the enzyme cannot be over-emphasized. Roberts's summing up [1941] of evidence on this point unfortunately lacks clarity. We admit that substrates like *p*-phenylene-

diamine and ascorbic acid, when added to fermented tea-leaf suspensions, are doubtless oxidized. But is it due to the existence of a cytochrome system? Is it not possible that the very reactive *o*-quinone reacts with these added substrates while it is itself being reduced to polyphenol? The enzymic nature of stimulation of O_2 uptake would thus be maintained and tea polyphenol would act as a carrier in the reaction. Thus the oxidations of ascorbic acid and *p*-phenylene-diamine would be only secondary reactions, and the fact that polyphenol-free enzyme preparations fail to oxidize directly these substances as well as quinol lends further support to this view.

Our enzyme preparations display a clear-cut specificity for *o*-dihydric phenols, and such a marked specificity is incompatible with the presence of cytochrome + cytochrome oxidase. However crude our enzyme preparation may be, it must be regarded as relatively pure when compared with tea-leaf mince and, unlike the latter, its reaction on substrates is free from the interference of tea polyphenols. To that extent, our results on substrate specificity of the oxidase system are more reliable than those of Assam workers.

Effect of organic solvents. Acetone inhibition seems to be characteristic of cytochrome oxidase, and so Roberts has inferred that our acetone-prepared enzyme would have undergone a substantial loss in activity. Nevertheless, we have obtained strong activity of this acetone-prepared enzyme on catechol and other *o*-dihydric phenols. The assumption that tea leaf originally contained cytochrome oxidase and that it was considerably inactivated during acetone treatment would be consistent with the failure of the enzyme to oxidize quinol and *p*-phenylene-diamine. But how are we to explain the strong activity on catechol unless, of course, it is granted that the leaf originally contained polyphenol oxidase also?

It is likely that besides cytochrome oxidase other plant oxidases are also inhibited to a different degree by acetone. For instance, Srinivasan [1936] has shown that ascorbic acid oxidase is inhibited by acetone. Although we have found the greater part of the original enzyme activity in our preparations, it is possible that they may not have retained the same polyphenol-oxidizing activity as in fresh leaf.

Oxygen tension. Further indirect evidence for the cytochrome theory consists in Roberts's observation that the initial rate of O_2 uptake by fermenting tea leaf is markedly accelerated by an increase in the O_2 tension. But it is doubtful whether this is an established property of cytochrome oxidase. Green [1940] states that 'the rate of oxidation of the ferrocytochrome by molecular oxygen in presence of the enzyme is practically independent of the partial pressure of O_2'. In fact, since the respiration of yeast and other micro-organisms is found to be independent of O_2 tension, this is taken as evidence for the functioning of cytochrome oxidase in aerobic systems.

Evidence for the cytochrome theory has also been inadequate on other points. For instance, the failure to depress the rate of O_2 uptake when catechol is added to fermenting tea leaf cannot be considered as proof for the carrier nature of the tea oxidase, as is shown in Part 5 of this series. The existence of both soluble and insoluble enzymes in tea makes any evidence based on solubility inconclusive. If the enzyme is considered too insoluble to be polyphenol oxidase, it is also too soluble to be cytochrome oxidase.

EXPERIMENTAL

Spectroscopic investigations

Presence of cytochrome in tea. Employing a microspectrophotometer we made observations on the finely ground tissues in presence of both sodium succinate and $Na_2S_2O_4$. Contrary to Roberts's results, examination of the basal portion of the stem in tea plant did not give any positive indications for the presence of cytochrome. Similarly, other tissues of the plant, when separately collected and tested, failed to display any characteristic absorption bands which could be identified with those of cytochrome.

No cytochrome could be detected in the acetone-extracted tea leaf from which the possible interfering substances like chlorophyll and tannin were removed by solvent extraction. Attempts to prepare cytochrome *c* from leaf by the method of Keilin & Hartree [1937] yielded negative results. Both Ceylon and Indian tea leaves were used in this investigation.

From certain considerations it seemed reasonable to expect the presence of cytochrome *c* in our enzyme preparations. First, Yakushiji's [1935] findings made the presence of cytochrome probable in the acetone-extracted tea leaf, from which our oxidase preparations were obtained. Secondly, the ammonium sulphate saturation employed in the preparation of cytochrome *c* from ox heart [Theorell, 1936] was also the method used for the purification of the soluble enzyme, as will be described later. Furthermore, since Roberts attributed the oxidizing activity of the enzyme preparations to the cytochrome system, it was reasonable to expect the presence of cytochrome *c* in highly active enzyme concentrates. But it was found that none of these preparations, when spectroscopically examined, displayed any specific absorption bands. Figs. 1 and 2 give the absorption spectra of a highly active tea oxidase solution. The results in the visible region of light (Fig. 2) were obtained in a Gaertner's polarizing spectrophotometer, while for a study of absorption in the ultra-violet region (Fig. 1) a Hilger's instrument was employed. 10 mm. cells were used in both cases.

It will be seen that there is no selective absorption either in the visible region or in the ultra-violet region studied, and therefore the preparation does not contain cytochrome. The component cytochrome a_3, which Keilin & Hartree [1939] identify with cytochrome oxidase, has a characteristic absorption (430 and 590 mμ), and most of the other haematins have a strong absorption in the extreme violet between 400 and 420 mμ [Dixon, Hill & Keilin, 1931]. But the spectrum of the tea-oxidase solution cannot be related to any of these.

Detection of cytochrome oxidase. The detection of cytochrome oxidase was based on its *in vitro* reaction on cytochrome *c*, which could be followed in the spectrophotometer. In view of the possible complications that would be introduced, the fresh-leaf mince or the finely ground tissue could not be used for this investigation. On the other hand, the acetone-prepared enzyme was considered a suitable material; for, if the observed activity of the preparation was attributed to the cytochrome system, it should be possible to demonstrate cytochrome oxidase in it.

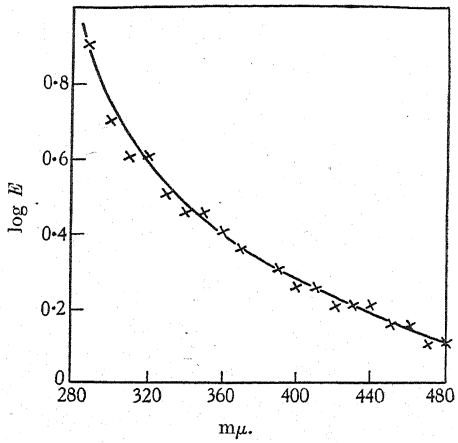

Fig. 1. Ultra-violet absorption spectrum of tea oxidase.

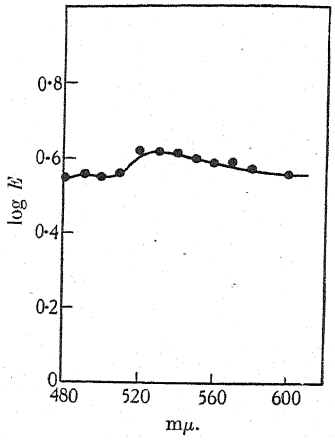

Fig. 2. Visible region absorption spectrum of tea oxidase.

Preparation of tea oxidase. In order to minimize the inactivating effect of the solvent on the enzyme, the acetone extraction of the oxidase was carried out at about $-20°$ in a bath of ice and salt under the following conditions, which are a refinement of those previously used by us. The leaf material was derived from young tea plants in the garden of the Indian Institute of Science, Bangalore. About 10 g. of fresh leaf were finely ground up, soon after plucking, with 30 g. of ice crystals and some iron-free sand and then introduced into a beaker which was kept immersed in a bath of ice and salt at $-20°$. Acetone (300 ml.), previously cooled to the same low temperature, was then added with vigorous stirring. The acetone extract was sucked off by means of an inverted thistle funnel, packed at the mouth with glass-wool, and immersed in the beaker. Renewal of cold acetone and repetition of the extraction three or four times rendered the preparation almost completely free of polyphenol, as tested by $FeCl_3$ and also by self-digestion of the preparation in the Warburg manometer. The material was quickly dried *in vacuo* and then extracted with 100 ml. phosphate buffer at pH 7·0, under cold conditions. After filtration the extract, which was highly active towards catechol, was brought to pH 5·0 with acetic acid and mixed with sufficient ammonium sulphate to saturate to the extent of 1/3. From the time of plucking up to this stage, when the enzyme may be expected to be more stable, the time taken was only 4–5 hr., and since the operations were, as far as possible, done in the cold, the inactivation of the enzyme must have been minimal during the preparation. The fluid was left overnight in the ice-box.

The slight precipitate that appeared was then filtered off, and to the filtrate more ammonium sulphate was added to bring it to half-saturation. There was a further precipitate which was also filtered off, and the filtrate was then saturated with ammonium sulphate, when again there was a precipitate. The three precipitates obtained were dispersed in water and dialyzed in the cold against running distilled water. The dialyzed suspensions, obtained by one-third or one-half saturation, contained for the most part inert matter with comparatively little enzyme, and they were therefore discarded. The precipitate at full saturation gave, on dialysis, a pale yellow solution constituting a very active preparation of oxidase. This could be preserved in the refrigerator for a considerable period without appreciable loss of activity.

The residue, after extraction of soluble enzyme from the acetone-prepared leaf powder, was further ground into a fine suspension and also preserved in the ice-chest.

Experiments with reduced cytochrome c and cytochrome oxidase from heart muscle. Cytochrome *c* was prepared by the method of Keilin & Hartree [1937], and it was found that goat's heart proved a better source than ox heart. The dialyzed cytochrome solution was preserved in the ice-chest. Reduction

of cytochrome was carried out by passing H_2 in presence of either freshly prepared palladinized charcoal [Hartung, 1928] or of platinized asbestos (E. de Haen's preparation). An active preparation of cytochrome oxidase was also obtained from fresh goat heart muscle by the method of Keilin & Hartree [1938].

The heart-muscle oxidase readily catalyzed the oxidation of reduced cytochrome, with consequent disappearance of the latter's characteristic absorption bands in the microspectrophotometer. But the tea-oxidase solution prepared as above, when added to the reduced cytochrome substrate, did not have any effect whatever on the absorption bands, which remained practically steady for about 24 hr. It must, therefore, be concluded that the specific cytochrome oxidase was absent from the tea-oxidase preparation. The activity towards catechol, on the other hand, was very marked, as determined by its rapid oxygen uptake ($-Q_{O_2} = 170$).

A suspension of the insoluble enzyme also gave similar spectroscopic results, which points to the same conclusion as in the case of the soluble enzyme.

The course of reaction of these enzyme preparations on reduced cytochrome, as compared with that of the heart oxidase, was further followed in the polarizing spectrophotometer. 0·5 ml. enzyme was allowed to react with 2·5 ml. reduced cytochrome solution (3×10^{-4} M) for 2 hr. at room temperature, and spectroscopic determinations were carried out in 10 mm. cells.

It will be seen from Figs. 4 and 5 that the absorption characteristics of reduced cytochrome remained unaffected in presence of tea-oxidase preparations, while heart oxidase (Fig. 3) brought about a complete change. In the case of heart enzyme and tea-insoluble enzyme the reaction mixtures had to be filtered before taking measurements in the spectrophotometer, and the slight shift in the curve in the latter instance is perhaps due to the absorption of cytochrome *c* on the solids of the tea-residue suspension.

The degree of oxidation of reduced cytochrome *c* is related to the fall in the intensity of its absorption bands. The kinetics of the oxidation can therefore be followed by measuring in the above reactions the extinction coefficients at 550 mμ, the maximum of reduced cytochrome *c*. Fig. 6 represents these data, and it will be seen that whereas the relative concentrations of reduced and oxidized cytochrome remain unaltered in the case of tea oxidase, the heart oxidase action results in a rapid fall in the reduced cytochrome concentration.

These results show that, judged on its specificity of reaction, tea oxidase cannot be identified with cytochrome oxidase.

Roberts [1941] has reported observing the reduced cytochrome bands when cytochrome *c* and succinate were added to the finely ground tea tissue and that these bands disappeared on vigorous shaking. In this reaction there was no need for any extraneous addition of cytochrome *c*, since he had

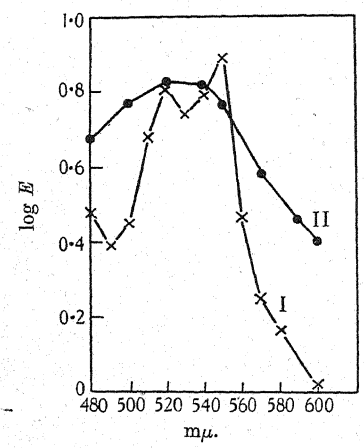

Fig. 3.

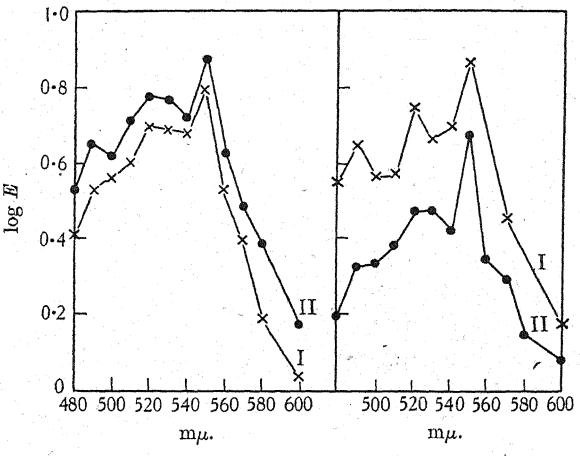

Fig. 4.

Fig. 5.

Fig. 3. Oxidation of cytochrome by heart oxidase. I, absorption spectrum of reduced cytochrome; II, reduced cytochrome *c* + heart-muscle enzyme.

Fig. 4. Action of tea-soluble enzyme on reduced cytochrome. I, reduced cytochrome; II, reduced cytochrome + tea-soluble enzyme.

Fig. 5. Action of tea-insoluble enzyme on reduced cytochrome. I, reduced cytochrome; II, reduced cytochrome + tea-insoluble enzyme.

already observed cytochrome in the tissue by its '*b*' band. Further, he has remarked elsewhere in his paper [Roberts, 1941] that when cytochrome *c* of ox heart is added to tea-tissue suspension it would combine with tea tannin and would thus be 'inactivated', thereby meaning perhaps an irreversible chemical change in cytochrome. If that is so, the disappearance of the reduced cytochrome bands may not be due to the presence of cytochrome oxidase. In our experiments we have found that

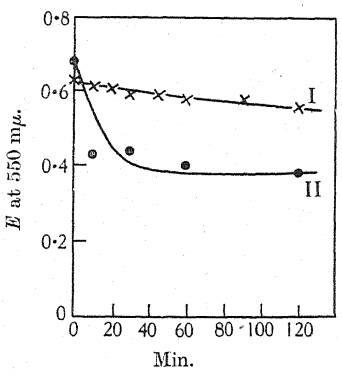

Fig. 6. Oxidation of cytochrome. I, tea oxidase; II, heart oxidase.

when reduced cytochrome is added to tea-tissue suspensions there is a rapid colour change from original red to a dirty yellow. The spectrum of the filtered liquid does not correspond to that of oxidized cytochrome and it cannot be reduced again either by Pd and H_2 or by $Na_2S_2O_4$. The change suffered by reduced cytochrome is not, therefore, a simple reversible oxidation, and under the circumstances the presence of cytochrome oxidase in the tissue becomes doubtful.

Manometric investigations

The oxygen uptake measurements were made in Warburg manometers with all necessary controls. All reactions were carried out at pH 7·0, total volume 2·5 ml., and temperature 30°. The first point established was the marked activity of the enzyme solution on catechol and its inactivity on reduced cytochrome *c* as substrate (Table 1).

Reduced cytochrome had not been reacted upon, as was shown by its absorption bands when tested at the end of the reaction.

In the *in vitro* reconstruction of the well-known respiratory system of cytochrome *c*, cytochrome oxidase, succinate and its dehydrogenase, it was desired to find out if the enzymes in the system could be replaced by tea-oxidase preparation. Results of oxygen uptake by the substrate, reduced

Table 1. *Oxygen uptake of tea oxidase with catechol and cytochrome*

Oxygen uptake when 0·5 ml. of diluted enzyme (original solution ×4) was allowed to react on 1 ml. of catechol solution (M/20) and 1 ml. of reduced cytochrome solution, respectively, in presence of 1·0 ml. of phosphate buffer at pH 7·0.

Time period (min.)	5	10	20	30	45	75	90	120	150
Catechol substrate (μl. O_2)	5	12	22	35	54	91	112	145	184
Reduced cytochrome substrate (μl. O_2)	3	1	4	1	0	0	0	0	4

cytochrome + succinate, in presence of tea-soluble and insoluble enzymes on the one hand and the heart enzyme on the other are given in Table 2.

Table 2. *Oxygen uptake of different enzyme systems on cytochrome c + succinate*

In the case of the heart enzyme the cytochrome concentration was $1·2 \times 10^{-5}$ M; in the case of tea enzymes $6·0 \times 10^{-5}$ M. 0·5 ml. enzyme and 0·5 ml. 1% succinate were used in all cases.

Time period (min.)	5	20	60	90	120	150
Tea-soluble enzyme (μl. O_2)	0	3	3	3	6	—
Tea-insoluble enzyme (μl. O_2)	0	4	8	6	3	—
Heart enzyme (μl. O_2)	4	—	46	59	90	110

Failure of tea enzyme to stimulate oxygen uptake in the above reactions may be due to the absence of either cytochrome oxidase or succinic dehydrogenase or both in the tea-oxidase preparations. If this mixture of reduced cytochrome *c* + succinate + tea oxidase is supplemented by a further factor, namely, succinic dehydrogenase free from cytochrome oxidase, oxygen uptake will then proceed, provided that tea oxidase contains cytochrome oxidase. But succinic dehydrogenase and cytochrome oxidase are invariably concomitant in animal tissues, and attempts to separate them by ultracentrifugation, heat inactivation and so on were not successful. However, if tea oxidase contains cytochrome oxidase, its addition to a succinic dehydrogenase preparation with only a weak cytochrome oxidase activity should result in an acceleration of the rate of oxygen uptake of the substrate mixture because of an increase in the cytochrome oxidase concentration. A succinic dehydrogenase preparation obtained from goat heart muscle by the method of Ogston & Green [1935] was found to have only a poor cytochrome oxidase activity, but strong succinic dehydrogenase activity, as tested by the usual methylene-blue technique. Addition of this preparation to the system 'reduced-cytochrome + succinate + tea oxidase' did not increase the rate of O_2 uptake in the reaction to any appre-

ciable extent. For the sake of clarity the comparative oxygen uptake values are summarized in Table 3.

Table 3. *Oxygen uptake by various reaction systems*

Reaction	Oxygen uptake μl./hr.
Catechol + tea oxidase	198
Reduced cytochrome *c* + tea oxidase	—
,, + succinate + tea oxidase	3
,, + ,, + succinic dehydrogenase preparation	9
,, + ,, + ,, ,, + tea oxidase	11

The absence of stimulation of oxygen uptake by the tea enzyme in all reactions in which cytochrome *c* formed the substrate must be interpreted as due to lack of cytochrome oxidase. The results obtained demonstrate unequivocally that tea oxidase cannot be identified with cytochrome oxidase. But, on the other hand, since catechol is readily oxidized by the enzyme, there can be little doubt about its polyphenol oxidase nature.

DISCUSSION

A similarity in a few properties led Roberts to propose his cytochrome theory of tea fermentation. Since then, these similarities have been shown to be spurious, and according to his own findings there are serious and 'very marked differences' between tea oxidase and animal-cytochrome oxidase. But these differences he attributes to a dissimilarity that may exist between plant-cytochrome oxidase and animal-cytochrome oxidase. Unfortunately, very little is known to date about plant-cytochrome systems, and until they are fully investigated it may be premature to base any conclusions on them.

In explaining the failure of ox-cytochrome *c* to accelerate tea-tannin oxidation, Roberts has gone even further. He has assumed tea-leaf cytochrome to be different from the ox-heart cytochrome in that the latter is inactivated by combination with tea tannin while the former is endowed with some type of immunity from tannin. If this be true it seems to be something specific to tea and perhaps to other tanniferous plants; for Brown & Goddard [1941] have observed that ox-cytochrome *c* does stimulate the oxidation of *p*-phenylene-diamine by the cytochrome oxidase present in wheat embryos. However, Roberts's postulate, of the presence in tea of a cytochrome immune to tea tannin, is reminiscent of his previous postulate of an immune peroxidase and protective substances, which has not as yet been confirmed.

Certain differences pointed out in the oxidase systems concerned in tea fermentation in Ceylon and Assam appear, on closer examination, to be a consequence of the use of different techniques in the two places. For instance, in our previous studies we have shown that catechol is oxidized by our enzyme preparations at a higher rate than tea tannin, and this has been confirmed. The results of Assam workers with their material appear to be exactly the reverse. The difference is only apparent, not real, because the conditions were not the same in both cases; the Assam experiments were carried out with minced leaf in which tea-tannin oxidation products had accumulated, but these were eliminated in our reaction mixtures. The tannin-oxidation products do interfere with the rate of subsequent oxidations by the enzyme, as is fully discussed in Part 5 of this series. Such an interference is in fact found to be responsible for the reversal in the rates of oxidation of catechol and tea tannin as observed by Assam workers. The suggestion of a difference in the nature of Assam and Ceylon enzymes receives, therefore, no support from the above consideration. A point of some interest in this connexion is that the present investigation also included the enzyme derived from tea plants raised on Indian seeds and grown under Indian conditions.

The spectroscopic and manometric evidence obtained in the investigation goes to show conclusively that tea oxidase cannot be identified with cytochrome oxidase, which forms the keystone of the entire structure of the cytochrome theory of tea fermentation. Absence of cytochrome further justifies the rejection of the theory. On the other hand, our original finding that the tea-fermentation enzyme was a polyphenol oxidase needs no modification whatever. The mechanism of tea fermentation based on this finding, whilst not so complex as the cytochrome theory, is consistent with the main facts so far observed.

SUMMARY

1. Attempts to demonstrate directly by spectroscopic methods the presence of cytochrome in tea have been unsuccessful.

2. Spectroscopic investigation of highly active tea-oxidase preparations showed that the enzyme could not be identified with cytochrome oxidase. Manometric results have confirmed this conclusion.

3. Some of Roberts's previous results have been reviewed in the light of the later work and certain

differences have been satisfactorily accounted for. From the evidence presented, Roberts's cytochrome theory of tea fermentation cannot be considered valid.

This investigation was carried out at the Indian Institute of Science, Bangalore, where spectroscope facilities were available. The author is indebted to Sir J. C. Ghosh, Director, Indian Institute of Science, Bangalore, for laboratory accommodation and to Mr M. Srinivasaya, Head of the Fermentation Technology Section at the Institute, for much helpful advice and discussion. Thanks are also due to Dr R. V. Norris, Director, Tea Research Institute of Ceylon, for permission to publish these results and to Mr J. Lamb, Biochemist, for his kind interest in the investigation.

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Studies on the 'Fermentation' of Ceylon Tea

7. THE PROSTHETIC GROUP OF TEA OXIDASE

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Earlier investigations in this laboratory on the specific nature of the oxidizing enzymes in tea [Lamb & Sreerangachar, 1940; Sreerangachar, 1943] have shown that the enzyme belongs to the family of polyphenol oxidases of which the potato oxidase and the mushroom oxidase are well-known members. The work of Kubowitz [1937; 1938] and of Keilin & Mann [1938] respectively on these enzymes has established that these polyphenol oxidases are Cu-protein compounds, and undergo a cycle of transformation from cupric to cupro form and vice versa, in the course of their catalytic activity. Cu is also found in the prosthetic groups of other oxidative enzymes such as laccase, ascorbic acid oxidase and tyrosinase, but the specific protein carrier in each of these enzymes is considered to be different. The Cu-proteins, unlike the Fe-porphyrin protein enzymes, show no specific absorption bands in the visible region of the spectrum, the absorption, if any, being only diffuse. A spectroscopic examination of tea oxidase has failed to reveal any characteristic absorption bands [Sreerangachar, 1943], and the enzyme is, therefore, analogous in this behaviour to Cu-proteins.

Consequent on the finding that tea oxidase was a polyphenol oxidase with respect to its substrate specificity, further investigations of the material and chemical nature of this enzyme became desirable, and it was thought that a study of the prosthetic group was a fruitful line to follow in the first instance. In particular, it was intended to see whether, like the other known examples of polyphenol oxidases, tea-polyphenol oxidase was also a Cu-protein compound.

Cu is easily detected in tea-oxidase preparations, but it was found that the crude preparations also contained Mn and Fe. As these two metals are also frequently associated with certain oxidizing enzymes, the possibility of their forming the active group of tea oxidase had to be considered in the early stages. According to Bertrand & Mallèvre [1895], there exists a close connexion between Mn and laccase, and Shaw & Jones [1935] have emphasized the importance of Mn in the study of the oxidative enzyme in tea. That the fresh tea leaf contains appreciable amounts of Mn can be seen by the fact that the ash is generally green because of the presence of manganous salts, which readily change over to the characteristic permanganate colour when wetted with oxidizing solutions. But in spite of this, the acetone-prepared enzyme contains only a fraction of the original amount (200–400 μg./g.). Further, Mn is almost completely absent even in partially purified preparations of enzyme and there-

fore it clearly cannot be associated with the active group of the enzyme.

The importance of Fe lies in the fact that, should it be possible to establish its constant association with tea oxidase, the latter would have to be classified along with Fe-containing enzymes or haemochromogens. This would not, however, be quite consistent with the chemical constitution of a polyphenol oxidase as understood at present. Tea-enzyme preparations do contain Fe, which not only persists in most of the purification stages, but also increases in proportion as purification proceeds. In this case, therefore, a more detailed examination was necessary, in order to ascertain if Fe was an essential component of the enzyme. It will be shown later that adsorption on tricalcium phosphate gel, and subsequent elution, completely eliminates all Fe from the enzyme, the activity of which is thereby enhanced. Fe is thus a non-essential constituent so far as enzyme activity is concerned.

Cu, on the other hand, is consistently present in all the purified enzyme preparations, which become richer in this element with each step in purification. A study, therefore, of the relation between the Cu content and the activity of purified tea-oxidase preparations would throw some light on the active group of the enzyme. The experiments described in the present communication provide evidence for the postulate that tea oxidase is a Cu-protein complex.

EXPERIMENTAL

As far as possible, particular care was taken to avoid any metallic contamination during preparation of enzyme and in the subsequent experiments. The use of sand in grinding leaf under acetone, as practised previously, was dispensed with in the present investigation. Cu-free distilled water was prepared by distilling ordinary distilled water successively with $KMnO_4$ and metaphosphoric acid in an all-glass apparatus. In the estimation of Cu and Fe, necessary corrections were applied for the small respective metallic contents of reagents.

Estimation of Fe *and* Cu

Fe was estimated by the α-α′-dipyridyl method of Shorland & Wall, [1936]. Digestions were carried out in 6 in. × 1 in. pyrex test-tubes with 1 ml. of conc. H_2SO_4 (Analar) and a few drops of Analar HNO_3. The digests were made up to 10 ml., 1 ml. of which was mixed in a $\frac{1}{4}$ in. Lovibond cell with 1 ml. of dipyridyl reagent and a trace of solid $Na_2S_2O_4$. The colorimetric determinations were carried out in a Lovibond tintometer, the red units being found proportional to the Fe content of the test material. With Fe solutions of known strengths, a standardization curve can be drawn (Fig. 1), and the Fe content of any unknown material can be calculated from its Lovibond readings by reference to the standard curve.

The Cu content of the preparations was determined by the colorimetric method of Keilin & Mann [1938], with sodium diethyl-dithiocarbamate reagent. Digestion of the material was carried out as above and 5 ml. digests were employed for the test. The CCl_4 solution was made up to 10 ml. and dehydrated by shaking with a small quantity

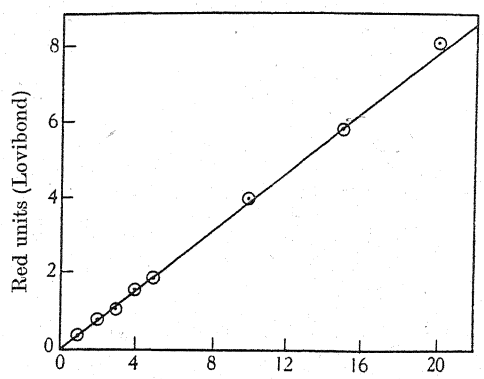

Fig. 1.

of anhydrous sodium sulphate. Colorimetric determination was again carried out in a Lovibond tintometer, with $\frac{1}{2}$ in. cells for all tests. The yellow readings are found to be proportional to the Cu content of the material as shown by Fig. 2, which was obtained by employing solutions of known Cu content. Measurements could be made correct to 0·1 yellow Lovibond units, corresponding to about 0·3 μg. of Cu in 10 ml. of CCl_4 test solution.

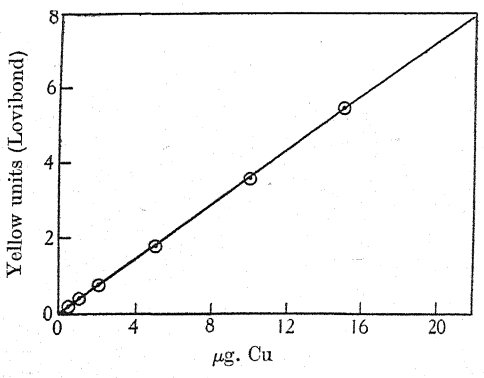

Fig. 2.

Total blanks were run for both the Fe and Cu contents of all reagents except the test solution, the procedure being identical with that of an experiment.

Estimation of enzyme activity

The activities of enzyme preparations were estimated by the method described in Part 4 of this series. Activities are expressed in terms of mg. ascorbic acid oxidized in 1 hr. at room temperature under the conditions described. Where necessary, activities are calculated/g. dry weight of the material.

Purification of enzyme

(*a*) *Extraction of enzyme from acetone-prepared leaf powder.* The acetone preparation of enzyme was obtained as described previously [Lamb & Sreerangachar, 1940] but without using sand for grinding leaf. It was observed previously that extraction of soluble enzyme from this acetone preparation varied with the reaction of the extracting medium up to pH 7·0. Alkaline reactions were not then tried. In the present experiment it has been found that the amount of enzyme extracted increases up to pH 10·0, beyond which the enzyme undergoes considerable inactivation. Sörensen's glycine buffer and Ringer's phosphate buffer were employed to obtain these alkaline reactions, and Table 1 gives results of extraction of 20 mg. acetone-prepared enzyme. After extraction the enzyme solution was adjusted to pH 5·0 with dilute acetic acid, and a fraction equivalent to 20 mg. of original powder was employed for activity determinations. All reactions were carried out in presence of McIlvaine's buffer at pH 5·0 and activity expressed in mg. ascorbic acid oxidized in 1 hr.

Table 1. *Enzyme extraction in alkaline solution*

pH ...	5·0	8·0	9·0	10·0	11·0	12·0
Insoluble enzyme activity	1·51	1·31	1·35	1·05	0·62	0·53
Soluble enzyme activity	0·98	1·02	1·38	1·56	0·32	0·05
Total activity	2·49	2·33	2·73	2·61	0·94	0·58

The unextracted powder gave an average activity of 2·60, which shows that up to pH 10 there is no enzyme inactivation. The amount of enzyme extracted is maximum (60 % of total) at pH 10·0, beyond which both the soluble and insoluble enzymes are considerably inactivated. It appears, therefore, that the most suitable reaction for extraction of soluble enzyme is pH 10·0, which was accordingly employed in the present investigation.

About 10 g. of the acetone-prepared leaf powder were extracted with 400 ml. buffer at pH 10·0 for 2 hr., filtered through muslin and squeezed by hand. The residue was extracted twice with 200 ml. buffer, the extract being squeezed out each time through muslin. The extracts were all collected, filtered through paper to give a clear reddish brown extract and adjusted to pH 6·0–6·5 by addition of dilute acetic acid. This acetone preparation had an activity of 0·21/mg. dry weight, while the activity of its extract was 0·43. If we define the amount of enzyme that oxidizes 1 mg. ascorbic acid as equivalent to a unit of enzyme, the crude extract was found to contain 966 units out of 2110 units of enzyme in the powder, i.e. about 46 % extraction.

(*b*) *Fractional precipitation with ammonium sulphate.* Solid ammonium sulphate was gradually added to the crude extract so as to give, as shown below, increasing concentrations of the salt. The precipitate appearing at each stage was filtered off before the next addition of $(NH_4)_2SO_4$.

Concentration of $(NH_4)_2SO_4$ (% saturation)	Resulting precipitate
5	No ppt.
10	No ppt.
25	Turbidity but no ppt.
33·3	Slight brown ppt. (A)
50·0	Thin dark ppt. (B)
75·0	Dark ppt. (C)
100·0	Dark ppt. (D)

Precipitates A–D were collected separately, suspended in water and dialyzed against distilled water until free of $(NH_4)_2SO_4$. They were all found to be completely tannin-free when tested with $FeCl_3$. The precipitates A and B, on dialysis, gave cloudy suspensions containing some insoluble matter, while C gave a brownish solution with very little insoluble substance. Precipitate D, on the other hand, yielded on dialysis a dark brown solution. Table 2 shows the activities and other particulars of these four fractions.

Table 2. *Properties of fractions obtained by* $(NH_4)_2SO_4$ *precipitation*

Fraction	Vol. of suspension (ml.)	Solid content (mg./ml.)	Activity (units/mg. dry wt.)	Enzyme content (units)
A	50	1·80	1·20	105
B	100	1·76	1·31	231
C	30	0·53	5·70	91
D	100	0·88	4·48	394

It will be seen that from an initial quantity of about 966 units a total of about 821 units of enzyme are recovered in the process. The fractions A and B seem to be similar in their activities and also in their solid-matter content. Most of the activity in these fractions appears to be associated with the insoluble matter; for the filtrates from A and B gave activities of only 0·42 and 0·41 unit/ml. respectively, while the original suspensions had activities of 2·17 and 2·31 units/ml. respectively. The dialysates resulting from fractions C and D had much higher activities than A and B, and they were, therefore, mixed and employed for further purification.

(*c*) *Purification with lead acetate.* In accordance with the procedure of Keilin & Mann [1938] for the purification of mushroom polyphenol oxidase, further purification of tea oxidase by means of lead acetate was attempted. In order to determine the amount of lead acetate required to remove impurities without affecting the enzyme, a trial experiment was conducted. 5 ml. portions of the dialysates (§ (*b*) above) were treated separately with 0·1, 0·2, 0·3 and 0·4 ml. 1/100 saturated lead acetate and the resulting

brownish gelatinous precipitate was immediately centrifuged off. The colour of the liquid diminished proportionately with the amount of lead acetate added, and in the last instance the centrifugate was almost colourless. The activities of the various solutions resulting from the above treatment are shown in Table 3.

Table 3. *Activity of material obtained by lead acetate fractionation*

Lead acetate solution added (ml.)	Activity of resulting solution (units/ml.)	Percentage of the original activity recovered
0	2·90	100·0
0·1	2·80	96·5
0·2	2·28	78·6
0·3	0·64	22·0
0·4	0·36	12·4

This preliminary test shows that lead acetate, even in small quantities, brings about a rapid inactivation of enzyme. On the dry weight basis, the activity of the solution resulting from the addition of 0·2 ml. lead acetate solution was 2·85 units/mg., which was only 70% of original activity. The method was, in consequence, useless for the purification of tea oxidase and was abandoned.

(*d*) *Fractional precipitation with acetone in the cold.* 40 ml. enzyme solution (§ (*b*) above) were cooled to −2 to −3° and mixed with cold acetone to give successively the following concentrations of acetone: (1) 5% acetone as employed by Keilin & Mann [1938] to remove readily precipitable impurities; (2) 40% acetone similar to Kubowitz's method [1938] for precipitating the potato oxidase, and (3) >80% acetone which effected more or less total precipitation. After good stirring the brown precipitates were centrifuged off, dissolved in a small quantity of McIlvaine's buffer at pH 7·0 and dialyzed against water.

Table 4. *Properties of the material obtained by fractional precipitation with cold acetone*

Conc. of acetone (%)	Vol. of fraction on dialysis (ml.)	Activity (units/ml.)	Dry matter (mg./ml.)	Activity (units/mg. dry wt.)
5	No ppt.	—	—	—
40	11	13·2	2·8	4·7
>80	9	6·4	2·3	2·8

The activity of the original solution is 5·0 units/mg. dry weight, and this value is not reached by any of the fractions obtained, although the 40% acetone fraction is very near to it. At 40% acetone concentration, therefore, the enzyme is concentrated into a smaller volume, but on the dry-weight basis no purification of enzyme has been achieved. Concentrations of acetone above 40% appear to bring about inactivation of enzyme. Thus acetone precipitation gave no more successful results in the purification of tea oxidase than the precipitation with lead acetate. (Table 4.)

(*e*) *Adsorption on calcium orthophosphate.* Calcium orthophosphate gel has been successfully employed in the purification of several oxidizing enzymes, e.g. mushroom polyphenol oxidase [Keilin & Mann, 1938], laccase [Keilin & Mann, 1938], and tyrosinase [Venkateswaran & Sreenivasaya, 1940]. Preliminary experiments on the use of this material for purification of tea oxidase gave promising results, and it has, therefore, since been adopted. In a typical adsorption experiment 150 ml. of $(NH_4)_2SO_4$-precipitated enzyme, containing 842 units, were acidified to pH 6·5 and mixed with 1·8 g. of freshly prepared gelatinous tricalcium phosphate. After standing for 30 min. the mixture was centrifuged and the colourless centrifugate, which contained only 42 units, was discarded. The phosphate cake was extracted three times with 10 ml. M/2 K_2HPO_4 solution, elution being carried out for ½, 2 and 4 hr. respectively. The mixture was centrifuged once again, and the eluates all combined and dialyzed against distilled water. 120 ml. of pale yellow solution resulted, with 0·53 mg. dry weight/ml. and an activity of 5·0 units/mg. dry weight.

A second adsorption as above resulted in 30 ml. of an almost colourless dialyzed solution: dry weight, 0·20 mg./ml. and activity 10·1/mg. dry weight. A 60–70-fold purification of enzyme in the acetone preparation (activity 0·15 unit/mg. dry weight) has thus been effected by $(NH_4)_2SO_4$ fractional saturation and the two adsorptions just described. This, taken in conjunction with the fact that the soluble enzyme forms, under the most favourable conditions, only 60% of the total quantity of enzyme present in the acetone preparation, shows that the actual concentration of soluble enzyme effected is about 100-fold. Further, on an average the acetone-prepared enzyme forms about 50% of the dry weight of leaf and the latter roughly amounts to a fourth of the fresh weight. These data, therefore, indicate that the activity of the purest preparation obtained is at least 800 times greater than the activity of fresh tea leaf.

There are indications that the stability of the enzyme suffers considerably on purification by adsorption, elution and subsequent dialysis. Most of the inactivation occurs during dialysis which, however, is necessary for the dry-weight determinations and therefore cannot be excluded. It is, therefore, advisable to neutralize the eluate and determine its activity before dialysis. Dry-weight determinations could be done on the dialyzed preparations, with proper allowance for the increase in volume during dialysis. Inactivation can be much reduced if the alkaline eluate is neutralized before dialysis. Thus it is possible that the protein carrier of the enzyme in a purer state cannot stand continuous contact with the alkaline medium, which may rapidly denature the protein. The possibility also of the existence of a protecting substance or an additional prosthetic group which is removed during adsorption cannot be excluded. The work of Keilin & Mann [1940] on the blue pigment of laccase lends support to the latter hypothesis.

Relationship between iron and copper contents and enzyme activity

In order to elucidate any possible relationship between Cu and Fe on the one hand and the activity of the enzyme on the other, quantitative data were obtained on the contents of these metals in the several purified preparations of enzyme as described above. Table 5 contains some typical results of these determinations and also the activities of the preparations/g. dry enzyme. To facilitate comparison, contents of Fe and Cu/unit activity of enzyme are also given.

Table 5. *Relationship between* Fe *and* Cu *contents and enzyme activity*

No.	Stage of purification	Activity (units/g. dry enzyme)	Cu content (μg./g.)	Cu content (μg./unit activity)	Fe content (μg./g.)	Fe content (μg./unit activity)
1	Acetone-prepared enzyme	151	32	0·21	150	1·00
2	Crude extract	330	60	0·18	119	0·36
3	$(NH_4)_2SO_4$ ½ sat. ppt.	890	145	0·16	1364	1·53
4	$(NH_4)_2SO_4$ full sat. ppt. I	3,100	267	0·09	2400	0·77
5	After adsorption on and elution from $Ca_3(PO_4)_2$ gel. I	5,060	625	0·12	0	0
6	$(NH_4)_2SO_4$ full sat. ppt. II	6,600	520	0·08	1558	0·24
7	$Ca_3(PO_4)_2$ adsorbed and eluted enzyme II	4,000	364	0·09	0	0
8	$(NH_4)_2SO_4$ full sat. ppt. III	3,546	335	0·09	789	0·22
9	$Ca_3(PO_4)_2$ adsorbed and eluted enzyme III, first adsorption	5,000	357	0·07	0	0
10	$Ca_3(PO_4)_2$ adsorbed and eluted enzyme III, second adsorption	10,100	800	0·08	0	0
11	Adsorbed cake residue from no. 9 eluted again with alkaline phosphate	2,830	313	0·11	0	0
12	Cake from no. 11 eluted with ⅕ sat. $(NH_4)_2SO_4$	2,440	244	0·10	610	0·25
13	$(NH_4)_2SO_4$ full sat. enzyme precipitated at 40% acetone concentration	4,700	714	0·15	1786	0·38

It will be noticed that Fe is present in the enzyme preparations up to a certain stage in purification but disappears as purification proceeds further. It actually increases during the initial stages, although this increase is not in proportion to the increase in activity. Absence of Fe in the adsorption-purified enzyme makes its association with the active molecule of the enzyme impossible.

On the other hand, there is a steady increase in the Cu content of the preparations, with increasing activity/g. dry weight of material. In the more active preparations, however, there exists a fair proportionality between enzyme activity and Cu content. The Cu content/unit activity approaches a constant value on progressive purification. When the activities of the purified enzyme preparations are plotted against their corresponding copper contents an approximate straight-line relationship becomes evident (Fig. 3).

There are two points which diverge very appreciably from the linear relationship between activity and Cu content shown by the rest of the data. Against these points the number of the preparation to which they refer is shown. No. 5 is a preparation in which the activity was determined after dialysis following adsorption and elution, in contradistinction to all the rest of the adsorption experiments, where enzyme activity was determined immediately after elution and neutralization. § 3 (*e*) above gives reasons for suspecting that the continued contact with alkaline phosphate inactivates the enzyme in its purer state, and for this reason the low value of enzyme activity here shown is also suspect. No. 13 refers to a fractional precipitation of the enzyme solution with 40 % acetone. It is not unreasonable to suggest that the acetone effected both a concentration of the enzyme (which would give a value much higher than the original 5·0) and also inactivated a portion of it. In this case the apparent drop to 4·7 would not represent the full extent of inactivation, and again, this result is rendered suspect. In both these cases, therefore, there are

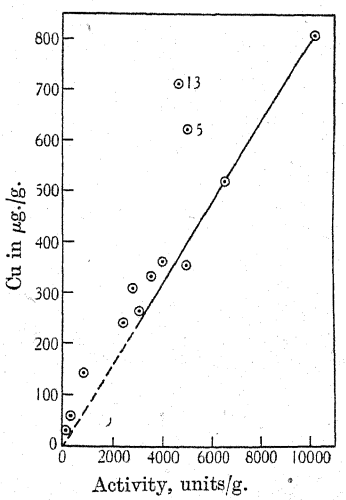

Fig. 3. Relationship between the Cu content and the activity of tea oxidase.

reasonable criteria for rejection of these two data when the activity-Cu relationship is considered.

The proportional relationship between the Cu content and the activity of the preparations is apparent even amongst the batches of acetone-prepared enzyme when such preparations are obtained from individual bushes whose leaf showed a fairly wide range of activity. For instance, the bushes whose acetone-prepared enzyme preparations had activities of 224, 193, 185 and 80 respectively/g. of material contained 20, 16, 16 and 4 μg. of Cu/g. respectively. Since even in these preparations, which can by no means be considered pure, the ratio between the enzyme activities and Cu appears to be of the same order as that obtained with further purified preparations, it is possible that the Cu found in these preparations is mostly enzymic. This idea receives support from the fact that some of the fractions exhibiting only slight activity do also contain some Cu, but it is not in any great excess over the amount that corresponds to their activity. However, not all the Cu present in the fresh leaf belongs to the enzyme. Part of the Cu present in fresh leaf but not essential to the enzyme is indeed removed during extraction with acetone.

Dialysis against KCN

While ordinary dialysis removes all 'free' or ionic Cu from the enzyme preparations, dialysis against cyanide results also in loss of bound Cu from the enzyme. This method of removal of bound Cu was originally devised by Kubowitz [1937; 1938] in his study of potato oxidase and later employed by others [Meiklejohn & Stewart, 1941; Allen & Bodine, 1941]. In the Cu-protein systems studied, complete removal of Cu resulted in total inactivation of enzyme, which showed that Cu formed part of the enzyme molecule.

In experiments with tea-oxidase preparations, dialysis against KCN has also been carried out, with a similar object. To 10 ml. samples of (*a*) $(NH_4)_2SO_4$-purified enzyme and (*b*) $Ca_3(PO_4)_2$-adsorbed enzyme, solid KCN was added to give a concentration of M/100 KCN, and the mixtures dialyzed against Cu-free water until all KCN was removed. In Exps. 2 and 3, the enzyme solutions were first dialyzed for about 4 hr. against M/1000 KCN solution and then against Cu-free water. Table 6 gives the activities and iron and copper contents before and after KCN treatment.

In the case of $(NH_4)_2SO_4$-purified enzyme there is apparently a tendency to retain part of the original Cu after treatment with KCN. By continued dialysis against KCN, as in Exp. 2, most of the Cu can, however, be removed from the enzyme solution. The enzyme preparation obtained after adsorption loses its Cu more easily when subjected to KCN dialysis, which renders this preparation completely free from Cu. It is significant that in every case the activity of the KCN-treated preparation is proportional to the amount of Cu retained in the solution; when the entire Cu is removed, the preparation becomes completely inactive in catalyzing the oxidation of polyphenols.

Table 6. *Dialysis of tea oxidase against* KCN

	Original enzyme	KCN-dialyzed enzyme	Percentage remaining after KCN treatment
Exp. 1. $(NH_4)_2SO_4$-purified enzyme			
Dialyzed against water			
Activity/g.	6000	2800	42
Cu, μg./g.	520	206	40
Fe, μg./g.	1558	1647	106
Exp. 2. $(NH_4)_2SO_4$-purified enzyme			
Dialyzed against KCN + water			
Activity/g.	2872	206	7
Cu, μg./g.	335	35	10
Fe, μg./g.	789	701	89
Exp. 3. $Ca_3(PO_4)_2$-adsorbed enzyme			
Dialyzed against KCN + water			
Activity/g.	3562	0	0
Cu, μg./g.	313	0	0
Fe, μg./g.	0	0	0

The Fe content is not significantly affected by the KCN treatment, and its presence seems to be immaterial for the activity of the preparations.

Previous workers, already cited, have found it possible, by addition of inorganic Cu, to restore the activity of preparations inactivated by dialysis against KCN. Kubowitz [1937; 1938] reports a restoration of 93%, Meiklejohn & Stewart 67%. The behaviour of tea oxidase, however, seems to be different in this respect from the above instances. For, when Cu is removed from tea oxidase by KCN dialysis, it has not been possible to restore activity even after addition of six to ten times the original amount of Cu. For this purpose the inactive dialyzed preparations were allowed, after addition of $CuSO_4$, to stand for a few hours and were then dialyzed against water to remove excess Cu. The preparations remained inactive.

The failure of inorganic Cu to restore the enzyme activity may perhaps be due to its inability, after once being split off, to recombine with the protein residue of the enzyme molecule. The possibly labile nature of the protein part has already been mentioned, and during KCN dialysis it is likely that the protein may have been altered in a manner which makes reconstruction of enzyme impossible. Even in Meiklejohn & Stewart's [1941] experiments, the necessity for adding large excesses of inorganic Cu to restore only part of the original activity may be considered as indicative of the unstable nature of

the protein residue of the enzyme. Although restoration of inactive tea-oxidase preparations has not been successful, the complete loss of activity that attends a total removal of Cu must be regarded as providing evidence for Cu forming the active group of the enzyme.

N *content of active preparations*

Since the purest enzyme is considered to be a Cu protein, its N content may be expected to be about 15–16 % of dry weight, as in the case of other proteins. N determinations of partially purified preparations may, therefore, be taken to indicate the extent of purification effected, provided the preparations do not contain any nitrogenous impurities. It seems unlikely that, after $(NH_4)_2SO_4$ precipitation and dialysis and the operations preceding it, the tea-oxidase preparations contain any unknown nitrogenous impurities. Table 7 gives the results of micro-determinations of N on some of the more active preparations of enzyme, together with their activities.

Table 7. N *contents of enzyme preparations*

Preparations	Activity (units/g. dry wt.)	N (%)
1. $(NH_4)_2SO_4$-precipitated enzyme I	4,700	3·96
2. $(NH_4)_2SO_4$-precipitated enzyme II	3,546	3·47
3. No. 2 after adsorption on $Ca_3(PO_4)_2$	5,000	4·97
4. No. 3 after a second adsorption	10,100	6·60
5. No. 3 elution with $\frac{1}{5}$ sat. $(NH_4)_2SO_4$	2,882	2·73

It will be noticed that the N content of the preparations varies with the degree of purification obtained. If the N content of the pure substance is about 15 %, our best preparation is only about 40 % pure.

DISCUSSION

In plant extracts, a certain amount of catalytic oxidation of ascorbic acid is probably due to the presence of ionic Cu, as distinct from the action of enzymic Cu, which is no doubt very much greater than the former. Since, in the present investigation, estimations of polyphenol oxidase activity have been carried out by means of ascorbic acid, it is relevant to consider if there has been any interference in the results from this source. Such interference, if any, is possible only in the case of acetone-prepared enzyme powder and the crude extract. All other preparations clearly cannot contain any ionic Cu, as they have all been subjected to a prolonged dialysis during the course of purification. The more important conclusions reached in these experiments have been based on the dialyzed preparations, and as such they are unaffected by any possible presence of Cu ions in the fresh leaf.

No correlation has been perceived between the Fe content and the activity of preparations studied. On the contrary, the most active preparations are characterized by a complete absence of Fe, while the Fe contents in other preparations rendered inactive by dialysis against KCN remained unaffected. The possibility, therefore, of the enzyme being of the nature of a haemochromogen is excluded. This is in support of our previous conclusions based on spectroscopic and other evidence.

On the other hand, the evidence presented in this investigation shows a close relationship between the Cu content and tea-oxidase activity. In the more concentrated preparations the catalytic activity runs fairly parallel to the Cu content. Removal of Cu from the enzyme complex by dialysis against KCN results in its inactivation. These results point to the conclusion that, like other known polyphenol oxidases, tea oxidase also is a metalloprotein with Cu as its prosthetic group.

To obtain an idea of the degree of success in the purification of the tea enzyme in our best preparation, a comparison may be made between the activities of our enzyme and that of Kubowitz. Calculated in terms of O_2 uptake, the purest preparation of tea oxidase obtained in these experiments has an activity of 27 μl. O_2/min./μg. of enzymic Cu ($Q_{O_2}/Cu = 1{\cdot}6 \times 10^6$), which is roughly one-tenth the figure obtained by Kubowitz for the potato oxidase. With the limited equipment available here, attempts to purify the enzyme further have not as yet proved successful.

SUMMARY

1. Tea oxidase has been purified to a concentration of at least 800 times that present in fresh leaf.

2. The purest preparation obtained had an activity of 10,100 units/hr./g. dry weight of enzyme, 1 unit of enzyme being defined as the amount which brings about the oxidation of 1 mg. ascorbic acid. The Cu content of this preparation is 0·08 % and nitrogen 6·60 %. It has been calculated that 1 μg. of enzymic Cu catalyzes an O_2 uptake of 27 μl. O_2/min.

3. The enzyme preparations purified by adsorption on $Ca_3(PO_4)_2$ gel were completely free from Fe.

4. In the more active preparations the polyphenol oxidase activity is proportional to the amount of Cu present. Complete removal of Cu inactivates the enzyme; resynthesis of enzyme by addition of $CuSO_4$ has not been possible.

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Oxidation of Acetic Acid in Animal Tissue

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Although it is known that acetic acid is readily oxidized in the animal, little is known of the intermediate stages on its oxidative path in the animal body, and numerous schemes have been suggested for its intermediate metabolism. In the present paper the oxidation of acetic acid and related 2-carbon compounds in animal tissue was examined. The results suggest that neither glycollic acid, glyoxylic acid, nor glycine are intermediates in the oxidation of acetic acid.

In the course of the investigation it was found that glyoxylic acid is a strong inhibitor of tissue respiration and of carboxylase. This observation may be useful in the study of enzymic reactions.

METHODS

Preparation of glyoxylic acid [Mohrschulz, 1926]. The Ca-salt was isolated and recrystallized three times (analysis: Ca, found 17·9%; calc. 17·9%). The Na-salt used was prepared from the Ca-salt by an equimolecular quantity of Na_2CO_3.

Manometric experiments were carried out at 40° in Warburg's conical flasks. Fresh tissue was either sliced or minced. In every experiment tissue from the same normally fed animal was used, and the results are therefore directly comparable.

The rate of disappearance of carboxylic acids was studied by a method first described by Meyerhof & Lohmann [1926] [see also Elliott & Schroeder, 1934]. The method is based on the fact that the oxidation of a carboxylic acid in a neutral buffered medium yields, under certain conditions, bicarbonate. The increase of bicarbonate in the solution is therefore a measure of the oxidation of the organic acid. Tissue slices (dry weight 7–30 mg. according to species and tissue) were suspended in 3 ml. physiological saline [Krebs & Henseleit, 1932] containing 80 μl. bicarbonate/ml. The substrates were added as Na-salts to the main compartment of the flask. 0·2 ml. 2N NaOH was placed into the centre well and, unless otherwise stated, the gas space of the cup was filled with O_2. The manometers were put into the thermostat and the O_2 uptake was measured after an equilibration period of 10 min. The total O_2 uptake was calculated by extrapolating for the equilibration period. After the incubation period (90–120 min.) the cups were cooled with ice, and the slices removed, washed, dried, and weighed. The NaOH in the centre well was replaced by 0·1 ml. 10% H_2SO_4, and 0·2 ml. 10% H_2SO_4 was measured into the side-arm. The gas space was filled with 5% CO_2 in O_2, and, after equilibration, the amount of $NaHCO_3$ in the solutions was estimated by acidification. The values found were corrected for the amount of $NaHCO_3$ carried by the tissue slices, on the assumption that a volume of liquid equalling 10 times the dry weight of tissue is introduced or removed with the tissue. From the values obtained, Q_{O_2} and $Q_{\text{bicarbonate}}$ ($Q_{\text{bic.}}$) were calculated. $Q_{\text{bic.}}$ is equal to the negative metabolism quotient of the substrate investigated ($-Q_{\text{substrate}}$), provided that no acid groups are formed during the experiment, and the carboxylic group is oxidized. When the carboxylic group is not altered as a result of the metabolism, the changes of bicarbonate found in the experiment are not related to the utilization of the substrate.

In experiments with minced tissue, 3 ml. of a suspension of tissue in phosphate saline [Krebs, 1933] were measured into the main compartment; 0·2 ml. 2N NaOH was placed into the centre well, and the substrate was measured into the side-arm and made up to 1 ml. with the saline medium. The gas space was filled with O_2. After an equilibration period of 10 min. the contents of the cup were mixed and the O_2 uptake was measured.

In anaerobic experiments, yellow phosphorus was placed in the centre well, the gas space filled with 5% CO_2 in N_2, and the CO_2 output measured.

In some of the experiments lactic acid was estimated according to Friedemann, Cotonio & Shaffer [1927].

RESULTS

Oxidation of acetate. As measured by the formation of bicarbonate, this is more rapid in guinea-pig kidney cortex than in any other tissue examined

(see Table 1): 0·0156M acetate (final concentration) increases Q_{O_2} about 50% above that of a control without substrate, and the average $Q_{bic.}$ is +7·6 as compared with +1·0 in absence of any substrate (average of 16 experiments). Elliott & Schroeder [1934] and Elliott, Benoy & Baker [1935] found in the presence of acetate a $Q_{bic.}$ of 4·7 in rabbit and 4·1 in rat kidney. $Q_{bic.}$ in the presence of acetate increases with the concentration of substrate (Table 2).

Table 1. *Oxidation of acetate in various tissues*

Tissue	Acetate added (M)	Q_{O_2}	$Q_{bic.}$
Guinea-pig: Kidney	0	−12·5	+2·0
	0·0156	−18·1	+7·3
Liver	0	− 6·4	+0·28
	0·0156	− 7·6	+2·27
Brain	0	− 8·0	+0·56
	0·0156	− 7·5	+1·15
Rat: Kidney	0	−23·2	+1·12
	0·0156	−29·6	+4·2

Table 2. *Effect of substrate concentration on the oxidation of acetate in guinea-pig kidney cortex*

Acetate conc. (M)	Q_{O_2}	$Q_{bic.}$
0	− 9·8	+0·68
0·00125	−17·2	+3·1
0·0025	−17·3	+4·0
0·005	−18·4	+5·5
0·01	−20·0	+6·4
0·02	−21·6	+7·2

Effect of pH. The effect of pH on acetate oxidation was studied by using gas mixtures containing different concentrations of CO_2 for filling the vessels. No NaOH was placed into the centre well. A higher rate of $NaHCO_3$ formation from acetate was found when the manometers were filled with a mixture of CO_2 and O_2, instead of O_2, with NaOH in the centre well. $Q_{bic.}$ slightly decreases with increasing concentrations of CO_2 in the gas mixture (Table 3).

Table 3. *Effect of* pH *on acetate oxidation*

Gas mixture	Solution in centre well	Approx. pH (calc.)	Acetate added (M)	Q_{O_2}	$Q_{bic.}$
O_2	NaOH	7·33	0	−12·2	+1·28
O_2	NaOH	7·33	0·015	−17·7	+5·91
1% CO_2 in O_2	None	7·33	0·015	—	+7·8
5% CO_2 in O_2	None	6·6	0·015	—	+6·9
20% CO_2 in O_2	None	6·0	0·015	—	+6·32

Effect of ammonium ions. Ammonium ions have a catalytic effect on the utilization of some substrates in kidney cortex [Edson, 1935; Krebs & Cohen, 1939]. Table 4 shows that NH_4Cl inhibits the oxidation of acetate, as measured by bicarbonate formation; the O_2 uptake is also reduced. No appreciable disappearance of NH_4^+ was observed. Inhibition of respiration by NH_4Cl has been previously observed in liver [Edson, 1935], in brain [Weil-Malherbe, 1938] and in the presence of acetoacetate in kidney [Edson, 1935].

Table 4. *Effect of* NH_4Cl *on acetate oxidation*

Additions (final concn.)			
Acetate (M)	NH_4Cl (μl.)	Q_{O_2}	$Q_{bic.}$
0	—	−13·5	+1·61
0·0156	—	−22·2	+9·15
0	1120	−13·0	+0·91
0·0156	224	−16·4	+4·35
0·0156	560	−17·5	+5·78
0·0156	1120	−17·2	+3·4
0·0156	2240	−17·6	+2·92

Effect of anaerobic conditions. No bicarbonate is formed from acetate under anaerobic conditions (Table 5). This confirms unpublished experiments of Dr D. Herbert [private communication], made by different methods.

Table 5. *Effect of anaerobic conditions on the bicarbonate formation from acetate*

Acetate added (M)	CO_2 liberated (μl.)	$NaHCO_3$ found (μl.)	Total CO_2 (μl.)
0	41	235	276
0·0156	39	228	267

Effect of malonate. Malonate was found to inhibit the formation of bicarbonate from acetate in guinea-pig kidney cortex (Table 6). The inhibition is slight at a concentration of 0·001M, whilst almost complete inhibition is found at a final concentration of 0·031M. This inhibition of acetate oxidation is abolished neither by glutamate nor by glucose. Inhibition of the oxygen uptake of liver slices in the

Table 6. *Effect of malonate on the bicarbonate formation from acetate*

Exp. no.	Additions: Acetate (M)	Additions: Malonate (M)	Q_{O_2}	$Q_{bic.}$
1	—	—	−15·1	+2·18
	0·0156	—	−22·4	+9·1
	0·0156	0·031	−13·5	+1·9
2	—	—	−15·7	0·0
	—	0·01	−13·0	+0·87
	0·0156	—	−23·3	+8·52
	0·0156	0·01	−14·8	+3·7
	0·0156	0·003	−17·2	+6·4
	0·0156	0·001	−17·8	+8·0

presence of acetate has been reported by Jowett & Quastel [1935].

Oxidation of 2-carbon compounds in kidney cortex. Challenger, Subramaniam & Walker [1927] suggested that in moulds acetate is oxidized to form glycollic, glyoxylic, and oxalic acids. This scheme of oxidation has also been suggested for animal tissue [Dakin, 1922; Toeniessen & Brinkmann, 1930]. The oxidation by kidney cortex of glycollic and glyoxylic acids and of two other 2-carbon compounds, glycine and ethanol, which might possibly be expected to be connected with the metabolism of acetate, was tested (Table 7). Neither glycollate, which was shown to be oxidized by rat- and rabbit-liver preparations [Dohan, 1940], nor glycine, is appreciably oxidized by guinea-pig kidney cortex. On the other hand, glyoxylate was found to be a strong inhibitor of tissue respiration. $Q_{bic.}$ of kidney tissue in presence of 0·0156M or lower concentrations of glyoxylate was generally negative. This observation indicates the formation of free acidic groups. Ethanol has no marked effect on the O_2 uptake; this confirms the results of Leloir & Muñoz [1938].

Table 7. *Oxidation of 2-carbon compounds in guinea-pig kidney cortex*

Exp. no.	Substrate	Q_{O_2}	$Q_{bic.}$
1	—	−14·3	+0·85
	0·0156M acetate	−20·4	+6·76
	0·0156M glycollate	−14·7	+0·99
2	—	−13·0	+1·85
	0·0156M acetate	−16·6	+5·15
	0·0156M glycine	−12·6	+1·82
3	—	−13·6	0·0
	0·0156M acetate	−20·2	+5·8
	0·0156M glyoxylate	− 7·2	−3·68
	0·0065M glyoxylate	−13·5	−1·5
	0·0032M glyoxylate	−14·5	−0·44
4	—	−12·2	+1·28
	0·0156M acetate	−17·7	+5·91
	0·0156M ethanol	−12·55	+1·12

Inhibition by glyoxylate. Glyoxylate has a strong inhibitory effect on the respiration of liver, muscle and kidney. In minced pigeon-breast muscle and liver the inhibition of respiration increases with time (Fig. 1; Table 8). The glyoxylate inhibition in pigeon-breast muscle is not abolished by citrate, fumarate or pyruvate, and only slightly by α-ketoglutarate or succinate.

Under anaerobic conditions, acid or CO_2 production of guinea-pig kidney cortex and of liver is increased by the presence of glyoxylate. No increased formation of lactic acid in the presence of glyoxylate was observed in liver tissue (Table 9). Glyoxylate therefore does not increase the glycolysis of liver, and it may be assumed that the formation of free acidic groups in the presence of glyoxylate under anaerobic conditions is due to a coupled oxido-reduction of the substrate according to the reaction:

$$2\mathrm{COOH.CHO} + \mathrm{H_2O} = \mathrm{COOH.COOH} + \mathrm{COOH.CH_2OH}.$$

Dismutation of glyoxylic acid according to the above reaction has been reported to occur in the presence of strong alkali [Böttinger, 1880], or of yeast maceration juice [Stepanow & Kusin, 1930].

Table 8. *Effect of glyoxylate on the respiration of minced pigeon-breast muscle and liver*

Cup	Tissue	Tissue wet wt. (mg.)	Glyoxylate (M)	O_2 uptake/60 min. (μl.)
1	Muscle	250	0	1179
2	Muscle	250	0·00625	441
3	Liver	400	0	865
4	Liver	400	0·00625	443

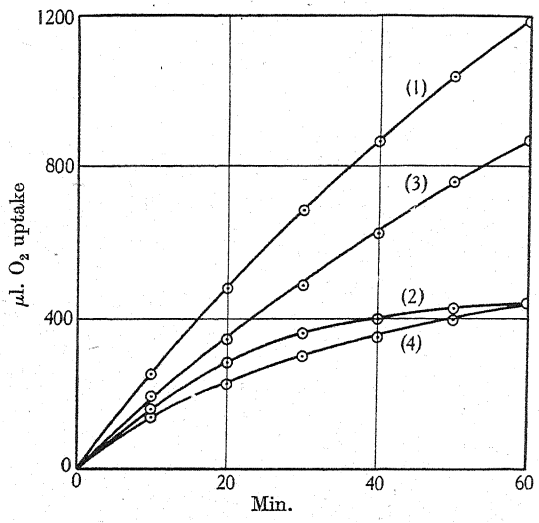

Fig. 1. The effect of 0·00625M glyoxylate on the respiration of minced pigeon-breast muscle and liver. The numbers of the curves correspond with the numbers of cups in Table 8.

Table 9. *Effect of glyoxylate on formation of* CO_2 *and lactic acid under anaerobic conditions*

No.	Tissue (of guinea-pig)	Glyoxylate (M)	Q_{CO_2}	Q_{lactic}
1	Kidney	0	1·39	—
		0·0143	3·13	—
	Liver	0	1·85	—
		0·0143	3·0	—
2	Liver	0	3·56	3·87
		0·0143	5·30	3·83

Effect of glyoxylate on carboxylase. The decarboxylation of pyruvate by fresh yeast maceration juice is inhibited by glyoxylate. This inhibition

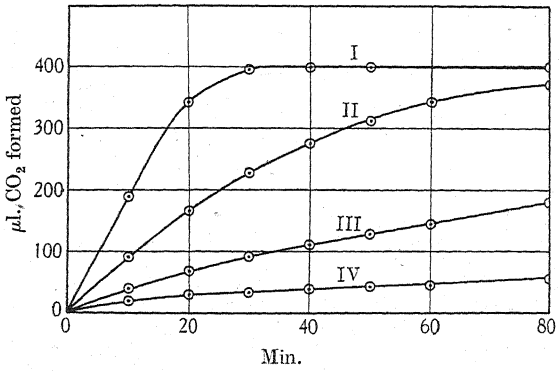

Fig. 2. Effect of glyoxylate on the decarboxylation of pyruvate by yeast maceration juice. Cup content: 400µl. pyruvate, 0·3 ml. 3M acetate buffer pH 5·0, 3·5 ml. H_2O. Side-arm: 1 ml. yeast maceration juice. Volume made to 5 ml., temp. 25°. Conc. of glyoxylate: I = 0; II = 0·001M; III = 0·002M; IV = 0·004M.

increases with increasing concentrations of glyoxylate (Fig. 2). It is probable that glyoxylate competes with pyruvate for the active groups of carboxylase.

DISCUSSION

The investigation of the oxidation of glycine, glycollic and glyoxylic acids in guinea-pig kidney cortex, as measured by the formation of bicarbonate, has shown that none of these substrates can be an intermediate in the oxidative path of acetate. Apart from the substrates investigated, the only substance that could arise by direct oxidation of acetic acid is oxalic acid, which is known to be little utilized in animal tissue [Dakin, 1922]. It seems that the oxidation of acetic acid follows other pathways, probably involving condensation reactions. Condensations of acetic acid have been demonstrated by Loeb [1912], who found that acetoacetic acid is formed from acetic acid, and recently Slade & Werkman [1942], using isotopic carbon, demonstrated the condensation of acetic acid to succinic acid and butylene glycol in bacteria. Lipmann [1941] suggests that acetate condenses with phosphate to form acetylphosphate before undergoing oxidation.

SUMMARY

1. The oxidation of acetate, glycollate, and glyoxylate in guinea-pig kidney cortex was studied by examining the effect of these substrates on the O_2 uptake and the formation of bicarbonate.

2. The oxidation of acetate, as measured by the formation of bicarbonate, was more rapid in guinea-pig kidney cortex than in any other tissue examined. On the average, 0·0156M acetate raised the Q_{O_2} by about 50% and $Q_{bic.}$ was +7·6 as compared with a $Q_{bic.}$ + 1·0 in absence of added substrate.

3. The oxidation of acetate was found to increase with the substrate concentration; it depends on the pH, the highest $Q_{bic.}$ having been found when the pH of the medium was decreased by filling the vessel with 1% CO_2 in O_2; NH_4^+ decreased the oxidation of acetate; no bicarbonate was formed under anaerobic conditions.

4. Malonate inhibited the oxidation of acetate in guinea-pig kidney. The inhibition was almost complete at a concentration of 0·031M malonate.

5. Glycine, glycollic acid, and ethanol were not appreciably oxidized in guinea-pig kidney. Glyoxylic acid is a strong inhibitor of tissue respiration.

6. Glyoxylic acid strongly inhibits the decarboxylation of pyruvate by fresh yeast maceration juice.

7. Glyoxylic acid to a small extent may undergo a coupled oxido-reduction in liver and kidney tissue to form glycollic and oxalic acids.

8. It is concluded that neither glycollic nor glyoxylic acid is an intermediate in the oxidation of acetic acid in guinea-pig kidney cortex.

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The Metabolism of Butyric and Related Acids in Animal Tissue

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Butyric acid is known to undergo a number of metabolic reactions in animal tissue, viz. oxidation to acetoacetic and β-hydroxybutyric acids, formation of carbohydrate [Cross & Holmes, 1937; Blixenkrone-Møller, 1938], and complete oxidation. The intermediate steps in these reactions are, however, still largely unknown. In the present paper experiments on the oxidation in animal tissue of butyric acid and a number of hitherto unexamined 4-carbon monocarboxylic acids will be reported. The metabolism of the latter was studied with the object of ascertaining whether they might be intermediates in the metabolism of butyric acid. It will be shown that vinylacetic, *dl*-αγ-dihydroxybutyric, γ-hydroxybutyric, and in some tissues *trans*-γ-hydroxycrotonic acids are oxidized in animal tissues; *dl*-βγ-dihydroxybutyric acid is little oxidized in kidney cortex, but causes the formation of a β-ketonic acid in starved rat and pigeon liver; tetrolic and tetronic acids are not oxidized in animal tissues, whilst vinylglycollic acid is an inhibitor of tissue respiration.

EXPERIMENTAL

Preparations

γ-Hydroxybutyric acid was prepared according to Fichter & Herbrand [1896]. b.p. 202°. The preparation was free from succinic acid.

Vinylacetic acid [Linstead, Noble & Boorman, 1933]. b.p. 72·5–75°/13 mm.; 39·5 mg. required 4·54 ml. 0·1N NaOH; calc. 4·60 ml.

Tetrolic acid [Feist, 1906]. m.p. 75·5°.

dl-αγ-*Dihydroxybutyric acid* [Nef, 1910]. The lactone of the acid, together with lactic acid, was extracted with ether from a mixture of saccharinic acids obtained by treating lactose with 8N NaOH. The acids were distilled at 5–6 mm., b.p. 115–120°; the distillate was neutralized with $NaHCO_3$, and the α-hydroxybutyrolactone was extracted with ether. The extracted lactone was converted into the phenylhydrazide (m.p. 130°), the hydrazide was decomposed with $Ba(OH)_2$, the phenylhydrazine extracted with ether and the lactone extracted after acidifying with H_2SO_4; 42·1 mg. required on heating 4·05 ml. 0·1N NaOH, calc. 4·04 ml.

dl-βγ-*Dihydroxybutyric acid* [Nef & Dar Juan, 1910]. 89·2 mg. required on heating 4·25 ml. 0·2N NaOH, calc. 4·37 ml.

trans-γ-*Hydroxycrotonic acid* [Rambaud, 1935]. m.p. 107·5°.

Vinylglycollic acid was isolated from the higher boiling fractions after the distillation of its methyl ester [van der Sleen, 1902]. b.p. 127·5°/11 mm.; 100·8 mg. required on heating 4·90 ml. 0·2N NaOH, calc. 4·95 ml. An attempt to isolate the crystalline Ba-salt [van der Sleen, 1902] was unsuccessful.

Tetronic acid was prepared by Dr H. A. Krebs according to Wolff & Schwabe [1896].

The other substrates were commercial preparations. The substrates were used as Na-salts.

Methods

1. The experiments were carried out on slices of kidney tissue from well-fed animals, unless it is stated that fasting animals were used. The oxidation of the various substrates was studied by the method of Meyerhof & Lohmann [1926] (see Kleinzeller [1943]). The formation of bicarbonate from crotonic and vinylacetic acids, as measured by this method, was compared with the disappearance of double bonds in the presence of these acids; the two methods were found to give similar results (Tables 6 and 7). As there are no specific methods available for estimating the other substrates, the assumption that the formation of bicarbonate is a measure of the utilization of these substrates is necessarily hypothetical.

2. Acetoacetic acid was estimated by the method of Edson [1935], which is specific for β-ketonic acids.

3. Estimation of crotonic and vinylacetic acids: The method is based on the estimation of H_2 necessary for the quantitative catalytic hydrogenation of the double bond. Both crotonic and vinylacetic acids are quantitatively hydrogenated under the conditions used for the estimation of aconitic acid [Johnson, 1939]. The conical manometric cups were filled as follows:

Main compartment: 2·0 ml. water, 0·5 ml. 5% H_2SO_4, 0·5 ml. catalyst suspension containing about 5 mg. Pd catalyst (Membranfilter Ges. Göttingen, 5% Pd on $BaSO_4$), finely ground and saturated with H_2.

Centre well: 0·1 ml. 20% NaOH, 0·1 ml. 20% pyrogallic acid, small glass bead.

Side-arm: 1 ml. test solution (acidified).

The cups were filled with H_2, and put into a bath at 40°. Equilibrium was quickly attained. Since no H_2 uptake was observed in the blank (without addition of substrate), it could be generally omitted. Recovery of added material was satisfactory (Table 1).

Table 1. *Estimation of crotonic and vinylacetic acids*

Acid added (μl.)	μl. H_2 uptake after 5 min.	10 min.	15 min.	20 min.
448 crotonic	381	447	448	446
*448 vinylacetic	410	421	421	421
*465 vinylacetic	405	450	455	456

* The somewhat low recovery of vinylacetic acid may be due to loss of this volatile acid during preparation of the solution.

In manometric experiments the following procedure was adopted: the contents of the cup were deproteinized with $\frac{1}{5}$ vol. of 5% HPO_3 and filtered; a measured portion was then transferred into a Kutscher Steudel extractor, acidified, and extracted with ether. The bulb of the extractor contained 1 ml. M $NaHCO_3$ to neutralize the extracted free acid and thus to prevent losses due to their volatility. The extraction was generally complete after 1 hr. (Table 2). After the extraction the ether was distilled off, and a measured portion of the acidified extract was used for the estimation.

Table 2. *Extraction of crotonic and vinylacetic acids*

(Volume of solution in extractor: 20 ml. Approximate rate of distillation of ether: 15 ml./min.)

Acid added (μl.)	Acid found (μl.) after extraction		
	0–30 min.	30–60 min.	Total
896 crotonic	881	16·5	897·5
834 vinylacetic	830	5	835

The method is not specific, for many unsaturated compounds, and also keto-acids, will be estimated in this way. The disappearance or formation of unsaturated compound will be denoted by the symbol Q_{Δ}, i.e. μl. H_2 absorbed/mg. dry weight of tissue/hr.

RESULTS

Oxidation of 4-carbon monocarboxylic acids in kidney cortex. It is known that kidney cortex oxidizes butyric, crotonic, β-hydroxybutyric, acetoacetic [Jowett & Quastel, 1935; Quastel & Wheatley, 1935] and *threo*-$\alpha\beta$-dihydroxybutyric acid [Weil-Malherbe, 1938; see, however, Hoff-Jørgensen, 1940].

In the present experiments the oxidation in guinea-pig and rat kidney cortex of various substrates at a concentration of 0·0156M was tested by means of measurements of the O_2 uptake and the bicarbonate formation; the results are summarized in Tables 3 and 4. It will be seen that butyric, crotonic, vinylacetic, β-hydroxybutyric, γ-hydroxybutyric, and *dl*-$\alpha\gamma$-dihydroxybutyric acids are oxidized in guinea-pig and rat kidney cortex. These substrates considerably increase Q_{O_2} and $Q_{bic.}$ above those of a control. *trans*-γ-Hydroxycrotonic acid is oxidized in rat kidney only. The following substrates are slowly oxidized in kidney slices of both species: *dl*-$\beta\gamma$-dihydroxybutyric acid, tetrolic, tetronic, and α-hydroxybutyric acids. Vinylglycollic acid inhibits tissue respiration, and $Q_{bic.}$ was found to be negative. The high rate of oxidation of γ-hydroxybutyric, *dl*-$\alpha\gamma$-dihydroxybutyric acid, and in rat kidney *trans*-γ-hydroxycrotonic acid, suggests that the hydroxyl group is oxidized with the formation of succinic, malic, and fumaric acids respectively. The recent observation by Lipmann & Perlmann [1942] that tetrolic acid is not attacked in kidney tissue is confirmed.

A study was then made of the rates of oxidation in rat kidney of butyric and related acids by measurement of Q_{O_2} and $Q_{bic.}$. In further experiments, again using rat kidney, as it oxidizes butyric acid at a higher rate than guinea-pig kidney (see Table 3), the disappearance of crotonic and vinylacetic acids and the formation of unsaturated compounds during the oxidation of butyric acid and β-hydroxybutyric acid were measured. As shown in Table 5, the latter acid is oxidized at a slower rate than the former, confirming the observation of Dakin [1922] on the behaviour of these substrates when administered to dogs. It seems unlikely, therefore, that β-hydroxybutyric acid can be an intermediate on the main pathway of the oxidation of butyric acid in rat kidney slices. It will be noted that vinylacetic acid was oxidized at a rate slightly higher than that of either butyric or

Table 3. *Oxidation of 4-carbon monocarboxylic acids in guinea-pig and rat kidney cortex*

Acid added as substrate (0·0156M)	Formula	Kidney cortex from: Guinea-pig No. of exps.	Guinea-pig $Q_{bic.}$	Rat No. of exps.	Rat $Q_{bic.}$
None	—	12	0·00–1·97	5	0·10–1·97
Butyric	$CH_3.CH_2.CH_2.COOH$	4	1·53–2·19	5	3·46–6·70
Crotonic	$CH_3.CH:CH.COOH$	4	1·98–3·17	3	2·31–5·59
Vinylacetic	$CH_2:CH.CH_2.COOH$	4	3·13–5·10	3	5·57–9·85
Tetrolic	$CH_3.C:C.COOH$	2	0·40; 1·84	1	−0·9
α-Hydroxybutyric	$CH_3.CH_2.CHOH.COOH$	1	1·77	—	—
β-Hydroxybutyric	$CH_3.CHOH.CH_2.COOH$	3	3·22–4·30	2	4·44; 4·82
γ-Hydroxybutyric	$CH_2OH.CH_2.CH_2.COOH$	7	2·75–4·20	3	5·20–8·10
trans-γ-Hydroxycrotonic	$CH_2OH.CH:CH.COOH$	2	1·21; 0·28	3	5·50–10·0
dl-$\alpha\gamma$-Dihydroxybutyric	$CH_2OH.CH_2.CHOH.COOH$	3	2·90–3·78	3	5·70–9·90
dl-$\beta\gamma$-Dihydroxybutyric	$CH_2OH.CHOH.CH_2.COOH$	4	0·30–3·10	1	1·4
Tetronic	$CH_2CO.CH_2.CO$ (O)	2	1·65; 2·43	1	−0·4
Vinylglycollic	$CH_2:CH.CHOH.COOH$	3	−0·35–−2·60	1	−3·2

Table 4. *Oxidation of 4-carbon monocarboxylic acids in kidney cortex*

Exp.	Substrate added (0·0156M)	Q_{O_2}	$Q_{bic.}$
Guinea-pig			
1	None	−14·3	+ 0·3
	Butyrate	−22·8	+ 1·57
	Crotonate	−22·3	+ 1·98
	γ-Hydroxybutyrate	−18·6	+ 4·2
	trans-γ-Hydroxycrotonate	−18·4	+ 1·2
	dl-βγ-Dihydroxybutyrate	−17·3	+ 3·1
	Vinylglycollate	− 7·2	− 0·35
2	None	−14·0	+ 1·15
	γ-Hydroxybutyrate	−16·6	+ 4·05
	dl-αγ-Dihydroxybutyrate	−14·35	+ 3·78
3	None	−11·2	+ 0·32
	Crotonate	−17·7	+ 2·06
	trans-γ-Hydroxycrotonate	−14·5	+ 0·32
4	None	−14·3	+ 0·32
	α-Hydroxybutyrate	−17·3	+ 1·77
	Tetronate	−14·8	+ 1·65
5	None	−12·2	+ 1·28
	Butyrate	−19·4	+ 2·19
	Tetrolate	−12·6	+ 1·84
6	None	−14·6	+ 0·75
	dl-βγ-Dihydroxybutyrate	−14·9	+ 1·08
	Vinylglycollate	− 5·7	− 2·6
7	None	−12·1	+ 1·67
	Vinylacetate	−22·1	+ 3·13
	dl-αγ-Dihydroxybutyrate	−16·7	+ 2·83
Rat			
8	None	−23·2	+ 1·12
	Butyrate	−32·2	+ 3·9
	α-Hydroxybutyrate	−22·6	—
	γ-Hydroxybutyrate	−27·7	+ 8·15
	trans-γ-Hydroxycrotonate	−25·2	+10·0
	dl-αγ-Dihydroxybutyrate	−25·7	+ 7·52
	dl-βγ-Dihydroxybutyrate	−25·9	+ 1·4
	Tetrolate	−15·6	− 0·9
	Tetronate	−26·5	− 0·4
	Vinylglycollate	− 5·8	− 3·2
9	None	−18·8	+ 1·97
	Butyrate	−29·4	+ 3·46
	Crotonate	−21·3	+ 2·31
	γ-Hydroxybutyrate	−29·9	+ 5·25
	trans-γ-Hydroxycrotonate	−24·6	+ 9·05
	dl-αγ-Dihydroxybutyrate	−27·4	+ 9·9

crotonic acid. Crotonic acid was generally found to be oxidized in rat kidney at a lower rate than butyric acid (see also Exp. 9, Table 4). According to these results vinylacetic acid could possibly be an intermediate in the oxidation of butyric acid in rat kidney but this needs to be ascertained by further investigations.

The rate of disappearance of double bonds in the presence of crotonic and vinylacetic acids is of the same order as the rate of bicarbonate formation from these acids (Tables 5 and 6). Butyric and β-hydroxybutyric acids yield an unsaturated compound, but as the method of estimating such compounds is not specific, no conclusions on the nature of the product formed can be drawn from this observation. Under anaerobic conditions vinylacetic acid disappears in rat kidney; Lipmann & Perlmann [1942] found under similar conditions a disappearance of crotonic acid.

The amount of unsaturated compound found when butyric acid is oxidized in rat kidney does not increase after the first hour, so that Q_Δ decreases, as shown in Table 7. As Jowett & Quastel [1935] have already shown, only small amounts of acetoacetic acid are formed in rat kidney in the presence of butyric acid, and the unsaturated compound formed is therefore unlikely to be acetoacetic acid.

Table 5. *Rates of oxidation of butyric and related acids in rat kidney*

No.	Substrate added (0·0156M)	Q_{O_2}	$Q_{bic.}$	Q_Δ
1	None	−22·8	+1·85	—
	Butyrate	−26·5	+6·70	—
	Crotonate	−25·2	+5·49	—
	Vinylacetate	−27·4	+9·85	—
	β-Hydroxybutyrate	−28·8	+4·82	—
2	None	−21·2	+0·12	+0·12
	Butyrate	−29·8	+5·15	+3·13
	Crotonate	−26·0	+3·36	−3·54
	Vinylacetate	−27·4	+5·57	−7·96
	(Vinylacetate; anaerobic	—	—	−2·89)
	β-Hydroxybutyrate	−28·8	+4·44	+1·92

Table 6. *Oxidation of vinylacetic acid in rat kidney*

(705 μl. vinylacetic acid added. The results refer to the whole period of the experiment.)

Duration of exp. (hr.)	Without substrate			With vinylacetate		
	Q_{O_2}	$Q_{bic.}$	Q_Δ	Q_{O_2}	$Q_{bic.}$	Q_Δ
1	−23·4	+1·88	+0·37	−29·1	+5·79	−6·30
2	−22·2	+1·43	+0·15	−27·7	+5·10	−4·35
4	−23·1	+1·80	+0·04	−27·7	+4·54	−5·45

Table 7. *Oxidation of butyric acid in rat kidney*

(The results refer to the whole period of the experiment.)

Duration of exp. (hr.)	Without substrate			With 0·0156M butyrate		
	Q_{O_2}	$Q_{bic.}$	Q_Δ	Q_{O_2}	$Q_{bic.}$	Q_Δ
1	−22·4	+0·39	+0·69	−32·6	+4·66	+6·0
2	—	—	—	−27·6	+4·08	+1·92
4	−20·4	+0·59	+0·26	−27·5	+3·39	+0·56

Effect of malonate. 0·031M malonate inhibits the oxidation of all the substrates examined in guinea-pig and rat kidney (Table 8). A slight formation of bicarbonate in the presence of malonate was observed in rat kidney when *trans*-γ-hydroxycrotonic acid was the substrate. The inhibition of the oxidation of butyric acid by malonate has already been reported by Jowett & Quastel [1935].

Table 8. *Effect of malonate on the oxidation of 4-carbon monocarboxylic acids in kidney cortex*

Exp. no.	Substrate added (0·0156M)	Substrate alone Q_{O_2}	Substrate alone $Q_{bic.}$	Substrate +0·031M malonate Q_{O_2}	Substrate +0·031M malonate $Q_{bic.}$
Guinea-pig					
1	None	−14·5	0·0	−10·2	+1·1
	Butyrate	−20·0	+2·1	−12·3	+0·9
	β-Hydroxybutyrate	−18·3	+3·3	−12·0	+1·4
	γ-Hydroxybutyrate	−20·0	+3·0	−11·0	+0·3
2	None	−11·4	+0·3	—	—
	Crotonate	−17·7	+2·1	− 9·3	+0·3
	trans-γ-Hydroxycrotonate	−14·6	+0·3	− 8·5	0·0
	dl-βγ-Dihydroxybutyrate	−13·7	+0·3	− 9·4	—
3	None	−12·9	+0·86	—	—
	Tetronate	−14·9	+2·43	−10·5	+0·41
	Tetrolate	−13·0	+0·42	− 9·4	+0·22
4	None	−14·3	+1·97	−10·6	+0·74
	Vinylacetate	−22·8	+3·34	−12·1	+0·78
	γ-Hydroxybutyrate	−19·3	+3·59	−11·9	+1·27
Rat					
5	None	—	—	− 7·1	−0·26
	γ-Hydroxybutyrate	−27·4	+6·9	− 7·2	−0·76
	dl-αγ-Dihydroxybutyrate	−26·7	+5·7	− 8·5	+0·17
6	None	−18·8	+1·97	− 8·4	−2·31
	Crotonate	−21·3	+2·31	− 9·6	−1·53
	trans-γ-Hydroxycrotonate	−24·6	+9·05	− 9·4	+1·97

Table 9. *Formation of β-ketonic acids from 4-carbon monocarboxylic acids in liver tissue*

(3 ml. phosphate saline; tissue; 0·0156M substrate; O_2; 60 min.)

Exp. no.	Substrate added	Q_{O_2}	$Q_{β\text{-ket. acid}}$
Pigeon liver			
1	None	−10·5	+0·55
	Butyrate	−11·1	+2·00
	Crotonate	−11·8	+2·2
	α-Hydroxybutyrate	−13·1	0·00
	β-Hydroxybutyrate	−12·3	+2·4
	γ-Hydroxybutyrate	−15·2	+0·95
	trans-γ-Hydroxycrotonate	−11·8	+0·65
	dl-βγ-Dihydroxybutyrate	−13·0	+1·22
	Tetrolate	−11·0	+0·85
	Tetronate	−11·1	+0·61
	Vinylglycollate	− 7·7	0·00
Rat liver			
2	None	−10·7	+0·82
	Butyrate	−12·6	+2·87
	Crotonate	−14·5	+5·58
	Vinylacetate	−14·6	+6·52
	dl-αγ-Dihydroxybutyrate	−13·5	+0·90
	dl-βγ-Dihydroxybutyrate	−13·4	+2·16
	γ-Hydroxybutyrate	−14·4	+0·82
3	None	−10·8	+0·75
	Butyrate	−12·8	+2·47
	Crotonate	−13·2	+4·43
	Vinylacetate	−13·6	+5·00
	β-Hydroxybutyrate	−13·1	+6·15

Formation of β-ketonic acids from butyric and related acids in liver. It is known that acetoacetic acid is formed in the liver from butyric, β-hydroxybutyric [Embden, Salomon & Schmidt, 1906], and crotonic [Friedmann, 1908] acids. Lipmann & Perlmann [1942] have shown that tetrolic acid does not give rise to acetoacetic acid in rat liver. A study was made of the formation, in the liver tissue of starving pigeons and rats, of β-ketonic acids from certain 4-carbon monocarboxylic acids not hitherto examined (see Table 9). β-Ketonic acids are formed from butyric, crotonic, β-hydroxybutyric, vinylacetic, and, to a small extent, from *dl*-βγ-dihydroxybutyric acids. None of the other substrates tested was appreciably ketogenic.

In the rat liver vinylacetic acid readily gives rise to acetoacetic acid. $Q_{acetoac.}$ exceeded, in our experiments, that from butyric and crotonic acids, but was lower than that from β-hydroxybutyric acid.

No β-ketonic acid is formed from vinylacetic or *dl*-βγ-dihydroxybutyric acid under anaerobic conditions (Table 10).

Table 10. *Formation of β-ketonic acids in starved rat liver under aerobic and anaerobic conditions*

Substrate (0·0156M)	O_2 Q_{O_2}	O_2 $Q_{β\text{-ket. acid}}$	N_2 $Q_{β\text{-ket. acid}}$
0	− 9·95	+0·86	0·00
Crotonate	−11·5	+3·08	0·42
Vinylacetate	−14·0	+5·85	0·15
dl-βγ-Dihydroxybutyrate	−12·5	+2·53	0·36

DISCUSSION

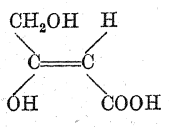
Blixenkrone-Møller [1938] found that β-hydroxybutyric acid does not give rise to carbohydrate in the perfused liver of cats, whilst butyrate does, and concluded that β-hydroxybutyric acid is not an intermediate in the formation of carbohydrate from butyric acid. He therefore suggested that butyric acid is oxidized in animal tissue both at the β-carbon atom, yielding ketone bodies, and at the γ-carbon atom, yielding succinic acid, which is then further oxidized.

The results of our experiments suggest that β-hydroxybutyric acid is not an intermediate on the main pathway of the oxidation of butyric acid in rat kidney. It would appear therefore that the formation of ketone bodies represents an alternative path in the oxidation of butyric acid in the animal. It is interesting to note, in view of Blixenkrone-Møller's suggestion of γ-oxidation of butyric acid, that γ-hydroxybutyric acid is oxidized at a higher rate than butyric acid in both guinea-pig and rat kidney slices.

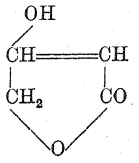
Under our experimental conditions, vinylacetic acid is oxidized in kidney slices at a higher rate than butyric acid. With vinylacetic acid a higher rate of β-ketonic acid formation than with butyric acid as substrate is also observed in starved rat liver. The compound thus shows physiological properties similar to those of butyric acid, and it seems possible that vinylacetic acid is an intermediate in the breakdown of butyric acid.

In our experiments a β-ketonic acid is formed in liver tissue from starving animals, in the presence of *dl*-$\beta\gamma$-dihydroxybutyric acid. Tetronic acid, which would be expected to arise from the oxidation of the β-hydroxyl of *dl*-$\beta\gamma$-dihydroxybutyric acid, does not behave as a β-ketonic acid (it does not yield any CO_2 on treatment with aniline citrate: personal communication from Dr H. A. Krebs) and consequently it cannot be the β-ketonic acid formed in the presence of *dl*-$\beta\gamma$-dihydroxybutyric acid.

The β-ketonic acid might be acetoacetic acid formed from substrates present in the liver tissue; such an effect would be similar to that observed when malonic acid is added to liver tissue [Jowett & Quastel, 1935]. *dl*-$\beta\gamma$-Dihydroxybutyric acid, however, increases Q_{O_2}, whilst malonate inhibits it. The possibility of the formation of acetoacetic acid from this substrate by dehydration seems unlikely in view of the observation that no β-ketonic acid is formed under anaerobic conditions from this substrate. On the other hand, it seems possible that a structural isomer of tetronic acid, hitherto unknown, may be formed, viz. the *trans*-isomer (I) of the enolic form of tetronic acid as opposed to the *cis*-isomer (lactone form, II):



I



II

SUMMARY

1. The oxidation, in kidney slices of well-fed guinea-pigs and rats, of the Na-salts of butyric, crotonic, vinylacetic, tetrolic, α-hydroxybutyric, β-hydroxybutyric, γ-hydroxybutyric, *trans*-γ-hydroxycrotonic, *dl*-$\alpha\gamma$-dihydroxybutyric, *dl*-$\beta\gamma$-dihydroxybutyric, tetronic, and vinylglycollic acids was studied by comparing the Q_{O_2} and the formation of bicarbonate in the presence of these substrates with that of a control experiment without substrate. Of the above substrates butyric, crotonic, vinylacetic, β-hydroxybutyric, γ-hydroxybutyric, *dl*-$\alpha\gamma$-dihydroxybutyric acids are rapidly oxidized in the kidney of both species. *trans*-γ-Hydroxycrotonic acid is oxidized in rat kidney only. Vinylglycollic acid inhibits tissue respiration. α-Hydroxybutyric, tetrolic, *dl*-$\beta\gamma$-dihydroxybutyric and tetronic acids are not appreciably oxidized.

2. From rates of bicarbonate formation in the presence of butyric, crotonic, vinylacetic, and β-hydroxybutyric acids in rat kidney slices, it is concluded that β-hydroxybutyric and crotonic acids are oxidized more slowly than is butyric acid. In the presence of vinylacetic acid $Q_{bic.}$ is higher than it is in the presence of butyric acid.

3. In rat kidney slices formation of an unsaturated compound in the presence of butyric and β-hydroxybutyric acids occurs under aerobic conditions.

4. Under anaerobic conditions vinylacetic acid disappears in rat kidney slices.

5. 0·031 M malonate inhibits, in the kidney of both species, the oxidation of all the substrates tested.

6. Vinylacetic acid and *dl*-$\beta\gamma$-dihydroxybutyric acid are ketogenic in liver slices from starved pigeons and rats.

7. It is suggested that vinylacetic acid may play a part in the intermediate metabolism of butyric acid in animal tissue.

I am very grateful to Dr H. A. Krebs for his constant advice and criticism during this investigation.

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Spectrographic Determination of Lead in Blood

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Although application of spectrographic methods of analysis to the study of the physiology of the 'trace elements' has been made in this country by Sheldon & Ramage [1931] and by Kent & McCance [1941], little study has been devoted to trace elements from the aspect of industrial metallic poisoning. In America, Cholak [1935] described a spectrographic method for the analysis of lead in blood, using bismuth as the internal reference element. This paper describes a spectrographic method adopted in the investigation of suspected industrial lead poisoning, and some of the results obtained.

METHODS

General. All analytical material was collected and prepared for spectrographing with strict precautions to prevent contamination, by ensuring that, where possible, the vessels and reagents used were Pb-free. Loss of analytical material was eliminated by reducing the handling and chemical treatment of the samples to a minimum.

Spectrograph. The large Hilger quartz spectrograph, which has a range of 1910–8000 A., was used with the standard conditions outlined in Table 1. For the Pb investigation the region 2700–4300 A. was employed. Identification of the lines was achieved by use of the Judd Lewis Comparator, an instrument which permits of simultaneous microscopic examination of the test plate and of a standard plate showing the position of all the elements. The line densities of emission spectra are in direct ratio to molecular concentration in a sample of their elements of origin. By use of the Hilger non-recording microphotometer to measure the ratio of the line densities of different known quantities of Pb, and a constant amount of an internal reference element, a graph can be prepared which provides a means of measurement of the unknown amount of Pb in a sample.

Table 1. *Standard conditions for spectrograph*

Wave band	2700–4300 A.
Slit width	0·015 mm. (fixed slit)
Arc gap	2 mm.
Voltage	120 d.c.
Amperage	5
Upper electrode	H.S. graphite pointed to 80° angle
Lower electrode	H.S. graphite 10 mm. diam. cut to form a crater to receive sample
Exposure	1 min.
Plate	Ilford H. and D. 70
Development:	(*a*) 90 sec. at 19·5°
	(*b*) Fixed, acid hypo 15 min.
	(*c*) Washed running water 15 min.
	(*d*) Dried in current of warm air

Standard of internal reference. In our earlier investigations the bismuth line at 2898 A. was chosen as the internal reference line for measurement against the Pb line at 2833 A., with satisfactory accuracy when checked by the addition of known quantities of Pb to duplicate samples of blood and synthetic ash. Investigation of subjects exposed to Bi of therapeutic origin confirmed our suspicions that Bi could be encountered normally in blood, and invalidated its use as a standard (Pl. 1, fig. 1).

After investigation of several elements, it was decided to use platinum as the internal reference element, since it fulfilled all the conditions required, and its line at 2830 A. is in a position of such proximity to that of Pb at 2833 A. as is ideal for measurement (Fig. 1). After experiment it was found that the most satisfactory Pt solution to add to the ash specimens, following solution in aqua regia, was one containing 0·01 g./100 ml. H_2SO_4. We consider it worth while to draw attention to the use of Pt in the analysis of several other elements implicated in poisoning by heavy metals. By varying the amount of Pt used, and if necessary the exposure time and type of plate in accordance with the spectrographic sensitivity of the element under investigation, Pt could be used as the internal reference element for the following metals:

As 2860·5 A.	Ni 3002 A.	Cr 2986·4 A.	Au 2676 A.
Pt 2830 A.	Pt 2998 A.	3015 A.	Pt 2702 A.
		Pt 2988 A.	
Th 2837 A.	V 3063·7 A.	Mn 2798·3 A.	Cd 2980 A.
Pt 2830 A.	Pt 3064·7 A.	Pt 2830 A.	Pt 2998 A.

With few exceptions all the above pairs of lines are those of *raies ultimes*. All the Pt lines are of high spectrographic intensity and are not overshadowed, which facilitates accurate measurement of line densities. Where the internal reference line is overshadowed by close juxtaposition of another element line, it may be difficult to be certain that the overshadowing line is not in part responsible for the deflexion on the galvanometer scale which is attributed to the internal reference line. Kent & McCance must have experienced this difficulty in their analysis of Au (2676·6 A.), with Mg (2795·5 A.) as the internal reference line. Consultation of Harrison's wave-length tables indicates a line of Fe at 2795·54 A., i.e. 0·01 A. difference.

Standard ash. The standard material used for preparation of the graph was a synthetic ash which gave a main base spectrum similar to that of blood. Persistent traces of Pb were found in all the supposedly pure chemical reagents used, even after prolonged refining by chemical and electrolytic methods. Spectrographic examination showed that the ash finally contained 1–2 μg. Pb/100 g.

To a number of samples of synthetic ash (0·05 g. the approximate weight of the ash of the blood samples used) different known amounts of Pb and a constant amount of Pt were added. The samples (duplicate) were then spectrographed. The curve from the mean of two sets of results was taken as the standard graph (Fig. 2). It is advisable to use the logarithmic ratios of the line densities, as smooth

working curves are obtained (Fig. 2). Some of the results from a series of identical samples used to check the accuracy of the graph are shown in Table 2. Errors of more than $\pm 8\mu g./100$ ml. of blood are exceptional, provided that the standard spectrographic conditions are strictly adhered to.

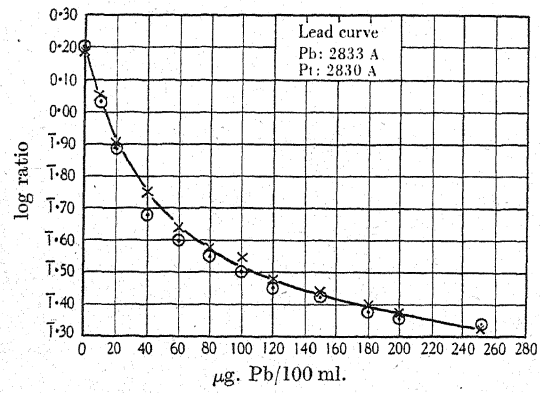


Fig. 2. Lead curve. Relation of spectrographic line intensity ratios to quantity of Pb. Ordinates, log intensity Pb 2833 A./Pt 2830 A.; abscissae, $\mu g.$ Pb.

Table 2. *Recovery of added lead*

Material analyzed	Ash weight (g.)	Pb added ($\mu g.$/100 ml.)	Pb found ($\mu g.$/100 ml.)	Error ($\mu g.$/100 ml.)
A. 5 ml. whole blood	0·0506	—	18	—
5 ml. whole blood and Pb	0·0522	40	60	+2
B. Whole blood	0·0516	—	17	—
Whole blood and Pb	0·0526	80	100	+3
Standard ash 1 + Pb	0·0500	50	48	−2
Standard ash 2 + Pb	0·0500	100	108	+8
Standard ash 3 + Pb	0·0500	120	116	−4
Standard ash 4 + Pb	0·0500	140	138	−2

Treatment of samples. 5 ml. of blood, urine, etc., were collected, transferred to a Pb-free silica crucible and sulphated by the addition of 3 ml. Pb-free H_2SO_4. The samples were then ashed in a dust-proof electric furnace by treatment at 110° for 12 hr., followed by 500° for 8 hr.

The low Fe content of serum, urine and cerebrospinal fluid gives spectrographically what is known as a weak base spectrum, which would be a source of error in applying the blood Pb curve to their analysis, as was pointed out by Foster, Langstroth & McCrae [1935]. We therefore added 0·02 g. of synthetic ash to samples of serum and urine; in this way their main base spectrum was intensified to resemble that of the standard samples. Experiment showed that the results obtained with serum and urine were accurate to $\pm 10\mu g./100$ ml. As yet we have not obtained sufficient quantities of cerebro-spinal fluid to check the application of the Pb curve; we assume that since its main base spectrum is similar to that of serum and urine, the error, when the synthetic ash additions are made, will be of the same magnitude. By this method it is possible to report results on 20 samples within 24 hr. after collection, with minimal use of chemical reagents and vessels.

RESULTS

In a series of 80 normal unexposed subjects we have found the mean concentration of Pb in the blood to be $40\mu g.$; the results ranged from 5 to $120\mu g./100$ ml. of blood, and the majority fell between 20 and $60\mu g.$

As was expected, those individuals who were exposed to the swallowing of metallic dusts, or inhalation of metal fumes, showed higher concentrations than office workers and the like. In four office workers, for example, the mean concentration was $16\mu g.$, compared with $88\mu g.$ in four metal workers. These latter findings are similar to those of Kehoe, Thamman & Cholak [1933*a*] and of Cholak & Bambach [1943].

The maximum normal concentration is the only thing that matters, since the concentration in an individual varies widely within normal limits from week to week. We have found, in sampling the same three individuals at intervals of four weeks, variations from $21–73\mu g.$, $150–53\mu g.$ and $43–11\mu g.$ respectively. One of us (A.T.) has found a similar variation in blood Pb concentration throughout the day. Although occupational exposure is mainly responsible for this variation in individual concentrations, the Pb content of the diet also contributes to it. From an analysis of foodstuffs we have found bread to contain $168\mu g.$ of Pb/100 g., tea biscuit $216\mu g./100$ g., sausage and cheese 85 and $43\mu g./100$ g. Some of the Pb content of these foodstuffs originates no doubt in contamination from vessels. That the Pb content of the diet contributes materially to blood Pb concentrations, conflicts with the view that there is little or no alimentary absorption of Pb by reason of the insoluble nature of the Pb compounds formed during the process of digestion. The total Pb in the circulation is, however, small in amount, and absorption of very small quantities may suffice to cause what is physiologically a substantial increase in its concentration in the blood. The following results (Table 3) show the effects on blood and urine concentrations of 12·5 grains lead

Table 3. *Effect of ingesting lead on the content of blood and urine*

	Subject	$\mu g.$ Pb/100 ml. before ingestion	$\mu g.$ Pb/100 ml. after ingestion
Blood	E.	11	250
	T.	14	155
	McE.	21	49
	B.	23	58
Urine	E.	5	107
	T.	3	72
	McE.	5	45
	B.	5	26

(5 ml. samples of blood or urine were analyzed.)

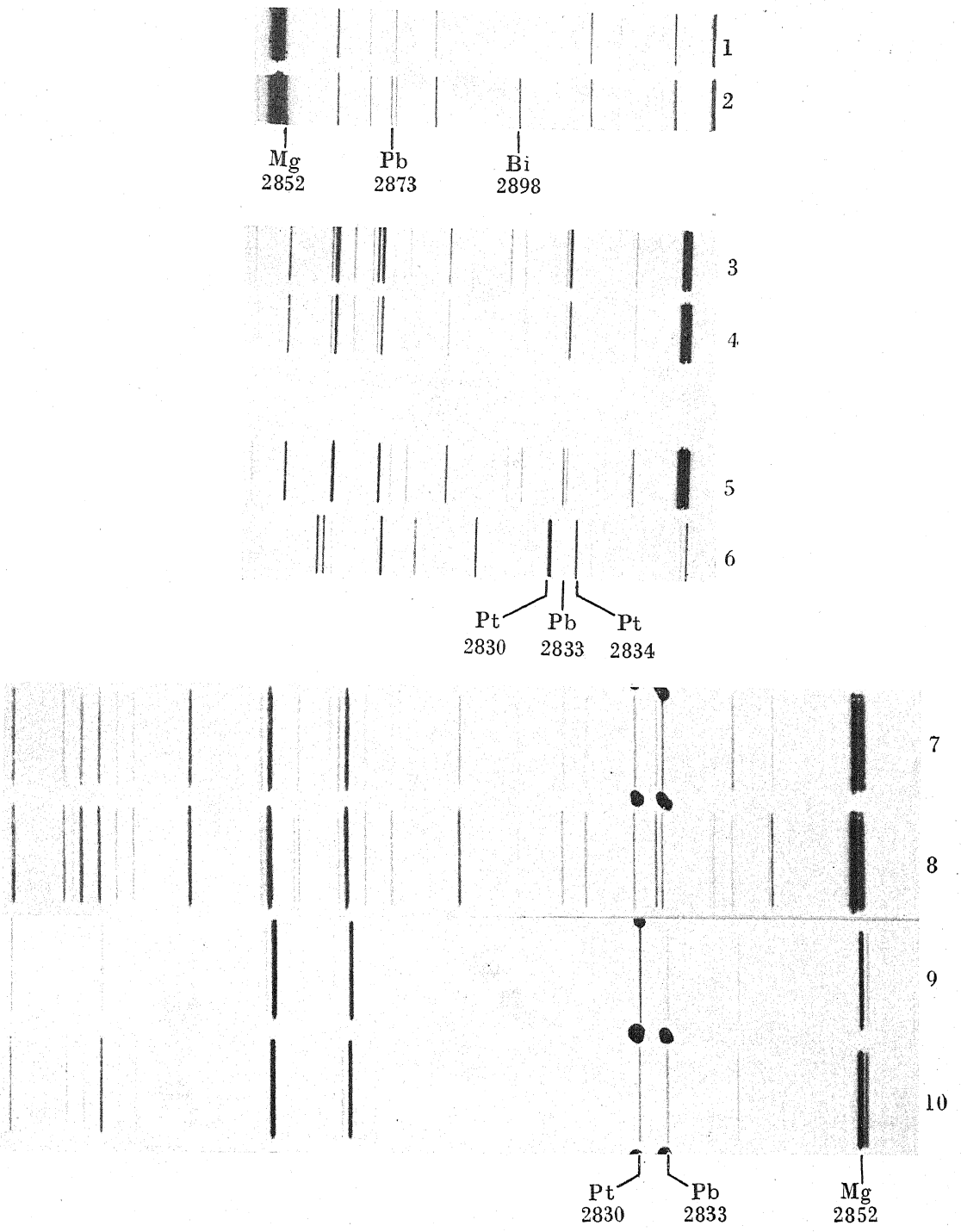
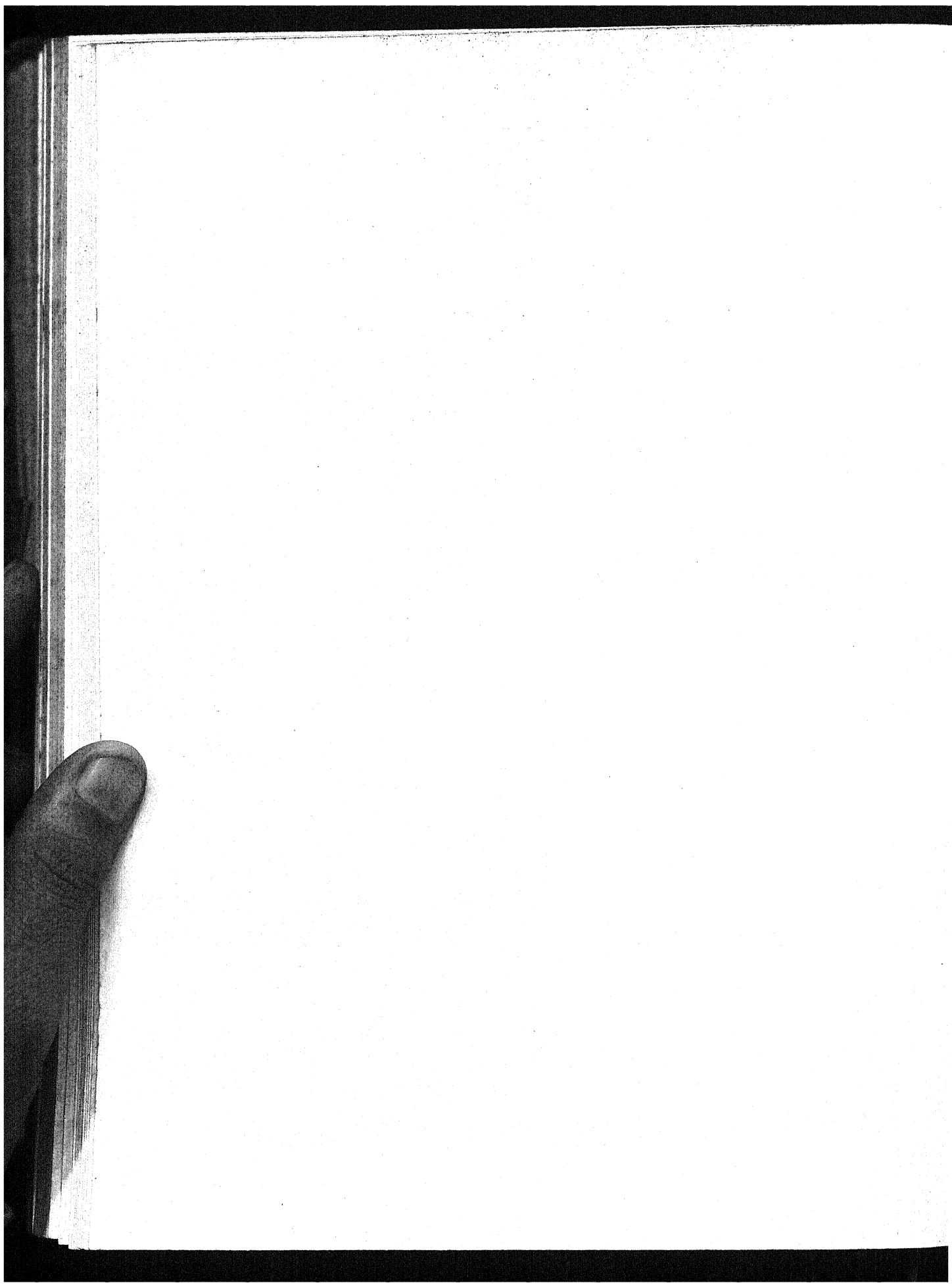


Fig. 1. Blood spectrograms. 1, shows Bi absent, while 2 denotes its presence due to absorption. 3, 4 and 5, blood spectrograms compared with a pure Pt spectrum 6 (note proximity of Pb 2833 A. to Pt 2830 A. and Pt 2834 A.). Variation in blood Pb content is evident from densities of Pb 2833 A. in 3, 4 and 5. 7 and 8, mother and infant bloods (case E). 9 and 10, infant urine and milk collected 5th day (case E); presence of Pb throughout clearly indicated, as also reduction of Fe lines in milk and urine.



acetate swallowed in the form of pills over three days by four subjects, who experienced no ill effects therefrom.

The bladder was emptied ½ hr. before and the urine for test collected ½ hr. after the blood was drawn. This gave an indication of the rate of excretion for a given blood concentration. From these results it is clear that considerable elevation of the blood Pb is possible by absorption of small quantities of Pb from the intestine.

Investigations on the blood of newly born infants shows that the Pb concentration approximates to that of the maternal blood. The investigation was carried out as follows. In the second stage of labour just before delivery 5 ml. of blood were withdrawn from the mother's arm, and 5 ml. from the umbilical vein prior to severance of the cord. The samples were covered and treated as described for spectrographing (A, B, C, Table 4, Fig. 1). In another similar experiment, samples of the maternal milk and urine from the infant on the fifth day (D, E, F, G, Table 4, Fig. 1) were analyzed.

Table 4. *Lead content of maternal and of infant blood, urine and milk*

	Lead (μg. Pb/100 ml.) in			
	Maternal		Infant	
Subject	Blood	Milk	Blood	Urine
A	54	—	57	—
B	43	—	42	—
C	34	—	28	—
D	46	32	71*	2
E	22	8	37	7
F	33	20	18	3
G	35	5	25	—

* Doubtful result: contamination probable.

DISCUSSION

Some diversity of results has been reported for normal blood Pb concentrations by different workers. Blumberg & Scott [1935], by visual comparison of the line densities, found the normal to vary from 5 to 100 μg./100 ml.; a critical concentration between 100 and 150 μg. produced symptoms. Kehoe, Thamman & Cholak [1935], using Bi 2898A. as the internal reference element, reported 58 μg./100 ml. to be the mean concentration in a series of 71 cases; this to us appears high. We agree with Blumberg & Scott that the maximum normal concentration is 100 μg./100 ml. and consider that, in cases of chronic exposure, the critical concentration is around 130 μg./100 ml. of blood. Although we agree with Tompsett & Anderson [1939], that the blood and urine Pb do not run parallel, it is evident that, in health, elevated blood concentrations are balanced by rapid excretion of the Pb absorbed. Our results further indicate that some control over diet and occupational exposure is necessary in the investigation of suspected Pb poisoning, and that errors in diagnosis may arise from single analyses. The Pb content of the diet, and occupational exposure, are, no doubt, accountable for some of the divergencies in results reported by different observers. One other possible factor in the variation of results is the Pb transmitted from mother to foetus. The amount is surprising, as also are the facts that the infant appears to retain it, and that the Pb content of the milk is sufficient to maintain the infant's original concentration. The results on human milk are higher than those reported by Kehoe *et al.* [1933*b*]. This may possibly be attributable to differences in time of sampling and sensitivity of the analytical method used. The unfailing appearance of Pb in blood and tissue fluids, and its transmission to the foetus, raises the question as to whether Pb has a physiological function as yet undiscovered. The opportunities for such investigations are few and collection of the necessary samples considerably more difficult than might be thought. In spite of the use of Pb-free crucibles and test-tubes for direct collection of milk and infant urine, occasional minute contamination is inevitable.

SUMMARY

1. A spectrographic method of lead analysis in blood is described, using platinum as the internal reference element.

2. Blood lead concentrations encountered in 80 healthy subjects ranged from 5 to 120 μg./100 ml. with a mean of 40 μg./100 ml. and greatest frequency of from 40 to 60 μg./100 ml. More than 50 % of the results lie between 20 and 60 μg.

3. Lead is transmitted to the foetus in amounts approximating to those of the maternal circulation.

4. Lead is present in human milk and is very slowly excreted in infant urine.

The authors are grateful to Dr D. McKay Hart, Maternity and Women's Hospital, Rotten Row, Glasgow and to Dr J. Eaton, Victoria Infirmary, Glasgow, for their co-operation and help in obtaining analytical material. To Mr J. Arnott, Weir Chemical Laboratory, they are also indebted for his criticism and encouragement, and desire to express their thanks to Sir John Richmond, G. and J. Weir, Ltd., Cathcart, Glasgow, for his permission to publish this article.

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The Vitamin B-complex Requirements of Several Insects

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It has been shown by us [Fraenkel & Blewett, 1943*b*] that six insects, which normally feed on flour and other dried food, grow satisfactorily on a diet of casein 50, glucose 50, yeast 5, cholesterol 1, McCollum's salt mixture 1, and water 15 parts, but entirely fail to grow when yeast is omitted. With the yeast replaced by yeast extract, the growth rate is considerably slower. No growth takes place if the diet contains a water-insoluble fraction alone in place of whole yeast. Previous work has indicated that insects require several vitamins of the B-group; the position up to 1940 has been reviewed by Craig & Hoskins [1940] and Trager [1941]. The present paper deals with the growth factors required by insects which are contained in the water-soluble and in the water-insoluble fractions of yeast. Short accounts of some of the results have already appeared elsewhere [Fraenkel & Blewett, 1942*a*, *b*; 1943*a*, *d*].

EXPERIMENTAL

The following beetles were used: *Tribolium confusum* Duv., Tenebrionidae; *Silvanus* (*Orycaephilus*) *surinamensis* L., Cucujidae; *Sitodrepa panicea* L., Anobiidae; *Lasioderma serricorne* Fab., Anobiidae; *Ptinus tectus* Boield., Ptinidae.

The following preparations and substances were used to compound diets:

Casein A/E, vitamin and fat free (Glaxo Laboratories Ltd.). Dried debittered brewer's yeast (Glaxo Laboratories Ltd.). Yeast extract, prepared from fresh brewer's yeast according to Chick & Roscoe [1930]. 'Insoluble yeast': residue from preparation of yeast extract, after 10 successive extractions with large volumes of boiling water.

Aneurin hydrochloride (B_1) (Glaxo Laboratories Ltd.), riboflavin (Glaxo Laboratories Ltd.), nicotinic acid (British Drug Houses Ltd.), pyridoxin (Glaxo Laboratories Ltd.), Ca-pantothenate (Glaxo Laboratories Ltd. and Roche Products Ltd.), choline chloride (British Drug Houses Ltd.), *p*-aminobenzoic acid (British Drug Houses Ltd.), *i*-inositol (British Drug Houses Ltd.), biotin methyl ester (Research Laboratories, S.M.A. Corporation), and biotin (Prof. du Vigneaud).

All the tests were performed in 2 × 1 in. specimen tubes. Each tube contained 2 g. of the dry diet and each diet was prepared in duplicate. Each test was performed with 20 larvae, 10 in each tube. The tubes were kept in a room of which the temperature was automatically maintained at 25° and the relative humidity at 70%. The criteria for assessing results are rate of growth from the newly hatched larva until pupation (*Tribolium*) or emergence of adult (other insects), and mortality. The methods of experimenting and assessing results are fully described elsewhere [Fraenkel & Blewett, 1943 *b*, *c*].

Preparation of a diet deficient in riboflavin. Yeast extract was treated with norit charcoal (Hopkin and Williams, Ltd.) according to the method of El Sadr, Macrae & Work [1940]. The yeast extract was not previously autoclaved, but as a precaution aneurin HCl was added to the diet, which then consisted of casein 50, glucose 50, cholesterol 1, yeast charcoal filtrate 15, insoluble yeast 5, McCollum's salt mixture 1 parts, aneurin HCl 50 μg./g. Riboflavin was added to the diet in graded doses and the results showing a graded response (Table 1)

Table 1. *Pupation of* Tribolium *on a diet deficient in riboflavin, with the addition of graded doses of riboflavin*

Riboflavin (μg./g. of diet)	No. of pupae	Spread in the pupation period (days)
None	11	60–113
0·625	16	50– 74
1·25	18	32– 43
2·5	16	29– 39
5·0	18	30– 39
10·0	16	30– 39
50·0	20	28– 39
Wholemeal flour (control)	19	27– 30

confirmed those reported by Fröbrich [1939], who grew *Tribolium* on a diet containing yeast extract which had been heated in an alkaline medium in order to destroy the riboflavin.

Substitution of yeast extract by pure vitamins. As pure vitamins of the B-group became available, attempts were made to feed mixtures of pure vitamins instead of yeast extract. Except in some earlier tests, wheat-germ oil was added to the diets

as a precaution against possible deficiencies of unknown fat-soluble factors. The vitamins were dissolved separately in a small quantity of water and then mixed so that 0·1 ml. of the mixture contained the precise amount required for each tube. The diets used in subsequent tests are recorded in Table 2.

Table 2. *Diets used in the later series of experiments*

		No. and composition of diet used in corresponding series of experiments					
		1	2	3	4	5	6
Casein (parts)		50	50	50	50	50	50
Glucose (parts)		50	50	50	50	50	50
Cholesterol (parts)		1	1	1	1	1	1
'Insoluble yeast' (parts)		5	5	5	5	5	5
McCollum's salt mixture no. 185 (parts)*		1	1	1	1	1 or 3	2
Wheat germ oil (mg./g.)		—	—	15	15	15	15
Water (parts)		15	15	15	15	15	15
Aneurin HCl	μg./g.	50	50	50	50	50	50
Riboflavin	μg./g.	50	50	50	50	50	50
Nicotinic acid	μg./g.	50	50	50	50	50	50
Pyridoxin	μg./g.	50	50	50	50	50	50
Ca-pantothenate	μg./g.	—	50	50	50	50	50
Choline chloride	μg./g.	50	50	50	50	500	500
p-Aminobenzoic acid	μg./g.	—	—	50	50	50	50
Inositol	μg./g.	—	—	—	50	500	500

* A modification of McCollum's mixture no. 185. Formula in Gay [1938].

RESULTS

Growth requirements of Tribolium confusum

The results with *Tribolium confusum* on different diets are recorded in Fig. 1. It is seen that addition

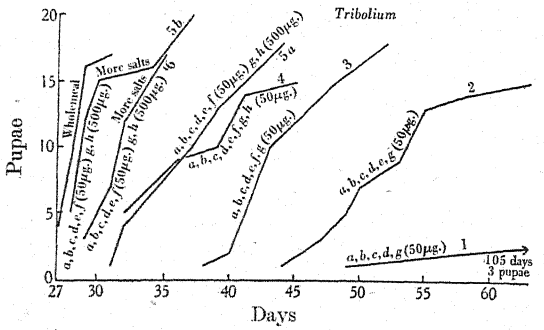

Fig. 1. Growth of *Tribolium confusum* on a diet of casein, glucose, cholesterol, 'insoluble yeast', salts and water, to which vitamins of the B-complex have been added in various combinations. Numbers on the curves refer to number of diet in Table 2. *a* = aneurin, *b* = riboflavin, *c* = nicotinic acid, *d* = pyridoxin, *e* = pantothenic acid, *f* = *p*-aminobenzoic acid, *g* = choline chloride, *h* = inositol. Quantities of vitamins given/g. of the dry diet.

of pantothenic acid in series 2 greatly improved the diet. In the third series *p*-aminobenzoic acid and wheat-germ oil, and in the fourth series inositol were added, again noticeably improving the diet. In view of the large amounts of inositol and choline required by vertebrates, these substances were given in amounts 10 times as large as those of the other vitamins in the fifth series. The full diet (Fig. 1, curve 5*a*) was now much better than the corresponding one of series 3 (curve 3), but still inferior to the control. With the quantities of each vitamin halved, the diet was not affected, probably indicating that the quantities were sufficient and also not large enough to cause toxic symptoms. Addition of more 'insoluble yeast' (15 % instead of 5 %) improved the diet somewhat, but a greater improvement was achieved by adding 1 % whole yeast. It is unlikely that this improvement was due to the supply of additional undetermined essential B-factors, because the same kind of improvement was obtained by approximately trebling the amount of salt mixture (cf. curve 5 *a* and *b*, Fig. 1). The diet now became virtually as effective as wholemeal flour. It therefore appeared that any missing factor supplied by 1 % yeast was a mineral.

A sixth diet was devised for *Tribolium* which contained twice as much salts as previously and it was compared with diets in which one of the vitamins was omitted at a time (Fig. 1, curve 6; Fig. 2). This diet proved of the same value as a diet which contained aqueous yeast extract in place of pure vitamins. In this series, but not in series 5, the diet was slightly inferior to wholemeal flour, but this difference is probably not significant. Without riboflavin, nicotinic acid, or pantothenic acid the diet

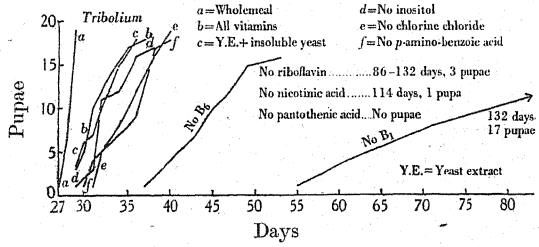

Fig. 2. *Tribolium confusum*. Growth on diet 6 (Table 2), compared with similar diets from which single vitamins have been left out one at a time.

became ineffective; without pyridoxin growth became slower, and still more so without aneurin. Omission of *p*-aminobenzoic acid or choline chloride produced a noticeable delaying effect, while absence of inositol impaired the efficiency of the diet only very slightly (Fig. 2).

All these diets contained a possible source of vitamins in the 'insoluble yeast' fraction which might have retained significant amounts of some factors even after prolonged extraction. Meanwhile the missing factor in 'insoluble yeast' had been found to be biotin (p. 689). It then became possible to perform, simultaneously with the tests of series 6, a parallel series in which 'insoluble yeast' was re-

placed by biotin (0·1 μg./g.) [Fraenkel & Blewett, 1943*a*, Fig. 3]. The results of these two series, one containing 'insoluble yeast' and the other biotin, were virtually identical, with three exceptions. In the biotin series absence of inositol delayed growth significantly, whereas absence of *p*-aminobenzoic acid had no effect. A more serious discrepancy applied to diets without pantothenic acid. These were totally inefficient in the 'insoluble yeast' series, but not quite so deficient in the biotin series, where 13 out of 20 larvae completed development, although at a greatly reduced rate. This discrepancy cannot at present be explained. It appears from a comparison of these two sets of tests that no significant amounts of vitamins could have been added to diets with 5% 'insoluble yeast'. Inositol is the only factor where the slight discrepancy in the results may, possibly, be due to retention of inositol in 'insoluble yeast'. An aneurin determination on our 'insoluble yeast' made by Dr Y. L. Wang (Cambridge) showed below 0·1 μg./g., a quantity too small to have any effect.

Growth requirements of other insects

Concurrently with the tests of diet 6 performed with *Tribolium*, the diets which contained 'insoluble yeast' were also tested with four other beetles. The results are summarized in Table 3.

The complete diet was as good, if not better, than the controls which contained yeast extract as a source of B-vitamins. Omission of inositol or *p*-aminobenzoic acid produced a slight, but possibly significant delay in growth. Absence of choline had a very pronounced delaying effect (Fig. 3), much greater than with *Tribolium*. Absence of the other vitamins had an effect similar to that described for *Tribolium*.

Lasioderma serricorne gave results quite different from *Tribolium* and *Ptinus*. Absence of riboflavin, nicotinic acid, *p*-aminobenzoic acid or inositol seemed to have no effect. Absence of aneurin, pyridoxin or pantothenic acid delayed development considerably, but to a much slighter extent than was the case with *Tribolium* and *Ptinus*. Absence of choline chloride had a very pronounced delaying action (Table 3, and Fraenkel & Blewett [1943*a*, Fig. 2]).

Sitodrepa panicea also gave results different from *Tribolium* and *Ptinus*, and from *Lasioderma*. The growth rate on the complete diet was considerably inferior to that of the control, where yeast extract was given instead of pure B-vitamins. Absence of nicotinic acid, inositol, choline chloride or *p*-aminobenzoic acid greatly affected the efficiency of the diet, which became totally inefficient only in the absence of aneurin (Table 3, and Fraenkel & Blewett [1944, Fig. 4]).

Table 3. *The effect on the growth of various Coleoptera larvae of the omission from the diet (Table 2, no. 6) of different members of the B-group of vitamins*

Description of diet	*Tribolium*			*Ptinus*			*Silvanus*			*Lasioderma*			*Sitodrepa*		
	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.
Wholemeal flour	114	27–29	19	85	57–67	16	115	25–29	15	94	35–39	20	97	37–41	17
Yeast extract + 'insoluble yeast'	100	29–36	18	100	47–58	18	100	27–35	16	100	34–39	19	100	37–39	15
All vitamins (Table 2, no. 6)	100	29–37	18	104	47–60	18	94	27–36	16	92	34–41	19	90	38–45	13
No aneurin	42	55–132	17	70	70–79	2	97	27–36	16	81	41–47	18	45	74–94	4
No riboflavin	31	86–132	3	0	—	0	94	30–46	9	97	34–41	18	88	41–49	9
No nicotinic acid	27	114	1	66	79	1	78	51	1	97	34–39	18	93	40–47	12
No pyridoxin	70	37–53	16	63	69–90	13	91	31–41	16	87	36–48	17	84	43–47	14
No pantothenic acid	0	—	0	70	69–77	2	103 (?)	30	1	87	36–41	8	93	39–49	5
No choline chloride	88	31–40	19	74	64–83	14	89	32–36	16	74	43–61	14	90	38–51	12
No inositol	97	29–38	17	98	48–58	17	91	29–37	15	97	34–41	18	90	41–45	13
No *p*-aminobenzoic acid	86	30–40	18	98	52–60	18	94	30–41	14	97	34–41	18	90	39–47	14

The growth rate (col. 1, under appropriate insect heading) is calculated as a reciprocal of the period in which half the number of insects completed their development and is expressed as a percentage of the controls. The period of development (col. 2) records the spread in the larval period (*Tribolium*) or larval plus pupal period (other insects). Col. 3 records the number, out of 20, completing development. The 'all vitamins' diet was that recorded in Table 2, no. 6. The relative growth rates of *Tribolium* and the other insects are not strictly comparable because they are based with *Tribolium* on the larval period only and with the other insects on the larval plus pupal period. Length of the pupal period of the insects in question is approximately 10 days.

Ptinus tectus [Fraenkel & Blewett, 1943*a*, Fig. 1] in the absence of single B-factors gave results almost identical with those described for *Tribolium*.

Silvanus surinamensis sometimes gave erratic results which could not be attributed to dietary factors. In the absence of aneurin, pyridoxin,

choline chloride, inositol or *p*-aminobenzoic acid, growth was not impaired. Absence of riboflavin caused high mortality and considerable delay in growth, and without nicotinic acid or pantothenic acid hardly any growth took place.

Effects of choline, inositol and p*-aminobenzoic acid*

To obtain a clear indication as to the nutritional importance of choline, inositol and *p*-aminobenzoic acid, a series of tests was devised for *Tribolium* and *Ptinus*, in which the diets contained these substances in various combinations. Diet no. 6, Table 2, was used. With *Tribolium* no difference was found in the efficiency of diets containing all the vitamins compared with diets lacking either inositol, or *p*-aminobenzoic acid, or both. Without choline chloride, the diet became considerably inferior, much more so than in the corresponding tests of Fig. 2. Pupation started after 37 days, as compared with 30 days on the 'all vitamins' diet. Omission of inositol + choline, or *p*-aminobenzoic acid + choline or of all three substances had no effect beyond that shown by the absence of choline.

With *Ptinus* the result was somewhat different (Fig. 3). Omission of inositol, or *p*-aminobenzoic

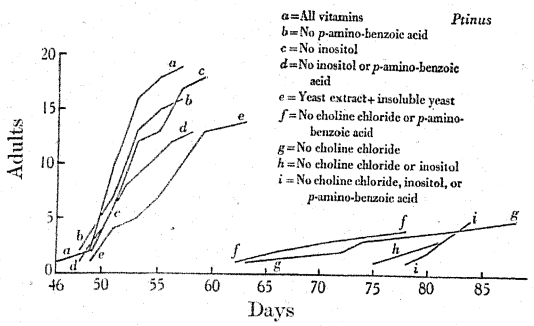

Fig. 3. *Ptinus tectus*. Explanations as for Fig. 2.

acid singly had a slight, but possibly not significant delaying effect, while a cumulative effect of the absence of both these substances became manifest. Without choline chloride, the diet became very inefficient. Omission of *p*-aminobenzoic acid + choline had no further effect, but without choline + inositol the diet was inferior to the 'no choline' diet. From these tests it appears that inositol is of some nutritional value for *Ptinus*.

Biotin as an insect growth factor

In the experiments so far described, with one exception (p. 688), the diets contained the insoluble fraction of yeast. Earlier experiments [Fraenkel & Blewett, 1943*b*] had shown that the water-soluble fraction of yeast was not fully equivalent to whole yeast, but had to be supplemented by the insoluble fraction, which was eventually found to be effective because it supplied biotin. The initial steps in the analysis which led to the recognition of biotin as the missing factor are described elsewhere [Fraenkel & Blewett, 1942*a*]. The result of a test with crystalline biotin methyl ester (25 μg. dissolved in 1 ml. of ethanol) is given in Fig. 4. The solution was taken up in a 1 ml.

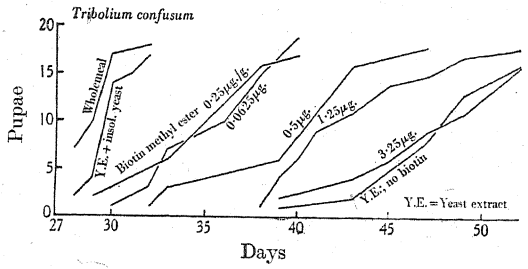

Fig. 4. Growth of *Tribolium confusum* on the yeast extract diet with the addition of graded quantities of biotin methyl ester.

hypodermic syringe and the quantities required were pipetted through the needle into the diets. The yeast extract diet was greatly improved by adding biotin methyl ester in quantities corresponding to 0·25 and 0·0625 μg./g. dry diet. In larger quantities biotin had a toxic effect. Quantities lower than 0·0625 μg./g. were not tested in this series. This very small dose produced an optimal effect which at first suggested that the optimal dose might be lower still. The toxic effects of larger quantities were not due to insufficient removal of the alcohol.

The experiment recorded in Fig. 4 was subsequently repeated with pure biotin in place of the methyl ester (Fig. 5). Growth was appreciably

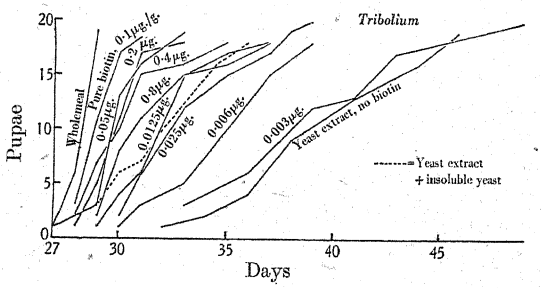

Fig. 5. Growth of *Tribolium confusum* on the yeast extract diet with the addition of graded quantities of biotin.

faster on the yeast extract diet without biotin than it had been on the former occasion. This is considered to be due to traces of biotin in the yeast extract of the Fig. 5 series. The optimum concentration of biotin was reached with 0·1 μg./g. dry food, 0·05 μg./g. being almost as efficient. Larger

quantities again appeared to be toxic, while lower quantities show a graded decreasing response with the threshold at the very low quantity of between 0·003 and 0·006 μg./g. This does not exclude the possibility that an optimal effect would have been shown with 0·1 μg. biotin methyl ester.

The difficulty of standardizing the yeast extract and getting consistent results made it desirable to construct a biotin-free diet which contained the vitamins of the B-complex as pure substances, instead of in yeast extract. The results of this series are given on p. 688 [cf. also Fraenkel & Blewett, 1943*a*, Fig. 3]. This diet, containing no yeast fractions, but 0·1 μg. biotin/g. of dry diet, is clearly as efficient as a diet which contains insoluble yeast as a source of biotin. Leaving out wheat-germ oil did not alter the efficiency of the diet, but without biotin growth became much slower and the efficiency of the biotin-free diet was about that of a typical yeast extract diet without biotin. The results of these tests showed that for *Tribolium* biotin is the only factor missing in a diet which contains yeast extract and no 'insoluble yeast'.

exception of *Silvanus*, omission of 'insoluble yeast' causes the diet to become deficient, and that the deficiency is made good by adding biotin.

A few tests were performed with the diaminocarboxylic acid which results from hydrolysis of the urea ring of biotin [du Vigneaud, 1942] and is capable of stimulating the growth of yeast in a biotin-free medium, exhibiting about 10% of the activity of biotin [du Vigneaud, Dittmer, Hoffmann & Melville, 1942]. For *Tribolium*, a biotin-free diet was considerably improved by the addition of 2·5 or 5·25 μg./g. of this diaminocarboxylic acid, but still remained considerably inferior to the same diet with biotin (0·1 μg./g.) added. 1 μg./g. or less of the diaminocarboxylic acid in the diet had no effect whatsoever (Fig. 6). These tests show clearly that biotin diaminocarboxylic acid has some effect in replacing biotin for *Tribolium*, but that, as with yeast, its activity is considerably less than that of biotin. This lesser activity cannot be compensated by using larger doses, because of a toxic effect.

All the tests so far described used diets containing glucose. On a yeast extract diet containing

Table 4. *Medium growth rate, period of development and number of pupae* (Tribolium) *or adults (other insects) formed, in the presence or absence of insoluble yeast or biotin*

(Explanation as for Table 3.)

Diet includes	*Tribolium* Growth rate	Days	Nos.	*Ptinus* Growth rate	Days	Nos.	*Silvanus* Growth rate	Days	Nos.	*Lasioderma* Growth rate	Days	Nos.	*Sitodrepa* Growth rate	Days	Nos.
Yeast extract + 'insoluble yeast'	100	29–36	18	100	47–58	18	100	27–35	16	100	34–39	19	100	37–41	17
Yeast extract + biotin	107	28–32	19	92	54–70	16	95	28–35	16	92	26–39	17	100	37–39	15
Yeast extract alone	80	32–46	19	83	56–70	15	95	29–36	14	69	41–64	14	91	39–51	13

Concurrently with these tests with *Tribolium*, corresponding tests were performed with four other beetles. The results are summarized in Table 4. It

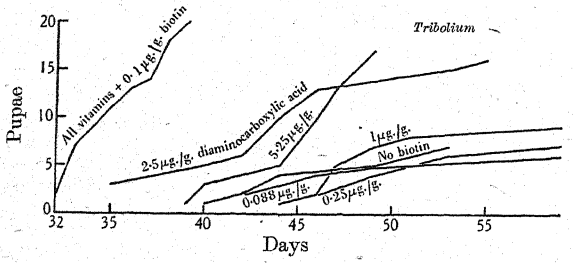

Fig. 6. Growth of *Tribolium confusum* on diets consisting of casein, glucose, cholesterol, salts, the eight members of the vitamin B-complex listed in Table 2, and in the presence or absence of biotin, or of graded amounts of 'biotin' diaminocarboxylic acid.

is clear [cf. also Fraenkel & Blewett, 1943*b*, Figs. 7–11 and Table 2] that for all the insects, with the

starch instead of glucose, *Tribolium* grew much better than in the diet containing glucose. The starch diet was almost as efficient as if it had contained 'insoluble yeast'. The effect was traced to the fact that even purified starch contained enough biotin to allow almost optimal growth of *Tribolium*.

DISCUSSION

A comparison of the effects of the absence of single B-vitamins on the different insects is given in Table 3. The most striking result is the difference in the nutritional requirements of *Tribolium* and *Ptinus* on the one hand, and *Silvanus*, *Lasioderma* and *Sitodrepa* on the other. The vitamin requirements of *Tribolium* and *Ptinus* are similar to those of the rat, dog and chick. Broadly speaking, aneurin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid are indispensable; in their absence growth is greatly affected. Choline and biotin are of somewhat lesser,

but still great importance, whereas inositol and *p*-aminobenzoic acid are of minor or perhaps no importance. With the other insects, the above-mentioned 'indispensable' vitamins seem to be less important, although their requirements proved very different in detail.

These two groups of insects differ in one important anatomical and physiological feature. *Lasioderma*, *Sitodrepa* and *Silvanus* belong to the large group of insects which contain intracellular symbionts [Buchner, 1930; Koch, 1933 *a*, *b*; 1936]. The latter seem to be absent with *Tribolium* and *Ptinus*. From the distribution of intracellular symbioses in insects several authors have considered their function to be provision of accessory food substances: Wigglesworth [1929], Buchner [1930], Aschner [1931], and Koch [1933 *a*, *b*] produced evidence in support of this suggestion for *Sitodrepa panicea*. This insect when freed of its symbionts will not grow on 'Erbswurst', an otherwise satisfactory food, but will grow on 'Erbswurst' plus 25 % yeast. Our results reported in this paper strongly support the view that intracellular symbionts are sources of vitamins. In their absence, with *Tribolium* and *Ptinus*, the 'indispensable' vitamins are necessary; in their presence *Lasioderma* grows well without any of them, *Silvanus* can do without aneurin and pyridoxin and *Sitodrepa* without nicotinic acid, pyridoxin and riboflavin. More recent experiments have shown that *Sitodrepa* and *Lasioderma*, when grown in the absence of symbionts, have vitamin requirements which are much more exacting than those of the normal larvae and resemble in every respect those of *Ptinus* and *Tribolium* [Fraenkel & Blewett, 1943*d*; 1944]. But even with *Tribolium* and *Ptinus*, in the absence of some of the 'indispensable' vitamins some growth takes place, and this may be due to partial intestinal synthesis. A parallel case is the synthesis of aneurin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid and biotin by micro-organisms in the stomach of ruminants [Wegner, Booth, Elvehjem & Hart, 1941; McElroy & Goss, 1941]. Intestinal synthesis of biotin in the rat has been demonstrated by Nielsen & Elvehjem [1942] and by Daft, Ashburn & Sebrell [1942], who produced symptoms of a deficiency, curable with biotin, by feeding rats with the bactericidal drugs sulphanilylguanidine and succinyl sulphathiazole.

Insects require essentially the same factors of the vitamin B-complex as rats, chicks and micro-organisms. *Drosophila* requires aneurin, riboflavin [van t' Hoog, 1935, 1936], nicotinic acid, pyridoxin and pantothenic acid [Tatum, 1939, 1941]; mosquitoes require the same substances [Trager & Subbarow, 1938; Subbarow & Trager, 1940]; *Tribolium* requires aneurin, riboflavin [Fröbrich, 1939; Offhaus, 1939], and nicotinic acid [Barton-Wright, 1941]; *Galleria* requires nicotinic acid [Rubinshtein & Shekun, 1941]. In none of these cases did the insects grow satisfactorily without the further addition of yeast or yeast fractions. Insects have also been shown to require for optimal growth in addition to yeast the water-insoluble fraction of yeast (Hobson [1933] for blowfly larvae, Fröbrich [1939] for *Tribolium*, and Tatum [1939, 1941] for the *Drosophila* larva). The need of *Tribolium* for biotin [Fraenkel & Blewett, 1942*b*] has been confirmed by Rosenthal & Reichstein [1942].

The need of certain insects for choline chloride now appears clear. In tests with *Tribolium*, omission of choline definitely decreases the efficiency of the diet. The need for choline was still more marked in experiments with *Ptinus* (Fig. 3) and most strikingly demonstrated with *Lasioderma* [Fraenkel & Blewett, 1943*a*, Fig. 2], in which choline seems to be the most important of the B-vitamins. With inositol and *p*-aminobenzoic acid the position is less clear and the results are conflicting, a position which closely resembles that experienced with vertebrates [György, 1942].

For *Tribolium* a diet without any yeast, but with the water-soluble B-vitamins and biotin in pure substance, with cholesterol and without wheat-germ oil, is in every respect as good as one with yeast or yeast fractions. This would appear to show that no further factors are required. *Ptinus*, *Sitodrepa* and *Lasioderma* also grow about equally fast with either insoluble yeast or biotin. All the insects, with the exception of *Sitodrepa*, grow as well on mixtures of pure B-vitamins as on yeast extract. Taking all the evidence together, biotin and sterols seem to be the only water-insoluble factors in yeast which are required by the insects in question. Also they do not seem to require any B-vitamins in addition to those already discovered (exception, *Sitodrepa*).

SUMMARY

1. Five insect species have been grown on an artificial diet which contains all the chemically identified members of the vitamin B-complex in pure form. For *Tribolium* and *Ptinus*, aneurin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid are essential vitamins; choline chloride is certainly, and inositol and *p*-aminobenzoic acid are possibly, of importance as growth factors.

2. *Lasioderma*, *Sitodrepa* and *Silvanus* do not seem to require some of the vitamins which are indispensable for *Tribolium* and *Ptinus*. This difference in the vitamin requirements is due to the presence in *Lasioderma*, *Sitodrepa* and *Silvanus* of intracellular symbiotic micro-organisms, which synthesize some of the vitamins.

3. When bred on a diet of casein, glucose, cholesterol, salts and aqueous yeast extract, insects

require, in addition, for optimal growth a substance contained in the water-insoluble residue of yeast, which was found to be biotin.

4. The optimal requirements of biotin for *Tribolium* are approximately 0·1 μg./g. of the dry diet, and the threshold of action is between 0·003 and 0·006 μg./g.

5. The insects tested do not appear to require any water-insoluble factor present in yeast in addition to a sterol and biotin.

6. The insects used, with the exception of *Sitodrepa*, grow on a mixture of pure B-vitamins as well as on yeast extract. It is concluded that these insects do not require B-vitamins beyond those already isolated.

We gratefully acknowledge a Government grant made by the Royal Society, which, partly, made this investigation possible. We are indebted to Mr A. L. Bacharach (Glaxo Laboratories Ltd.) for providing us with liver extract, pyridoxin and Ca-pantothenate; to Roche Products Ltd. for a gift of Ca-pantothenate; to Prof. V. du Vigneaud (New York) for a gift of biotin and biotin diaminocarboxylic acid; to Dr J. H. Sang for a sample of biotin concentrate (Research Laboratories, S.M.A. Corporation); and to Drs H. Chick and T. F. Macrae (Lister Institute of Preventive Medicine) for some invaluable advice.

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The Sterol Requirements of Several Insects

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It has been shown [Fraenkel & Blewett, 1943*a*] that six insects which normally feed on flour and other stored food grow satisfactorily on a diet of casein 50, glucose or starch 50, yeast 5, cholesterol 1, salts 1 and water 15 parts. Without cholesterol, growth became slightly (*Tribolium, Silvanus, Lasioderma*), appreciably (*Ptinus*) or severely (*Sitodrepa, Ephestia*) impaired (Table 2 (1)). If the diet contains aqueous yeast extract in place of yeast together with cholesterol, all the insects develop, though at a reduced rate resulting from a deficiency of biotin. Without cholesterol the diet becomes entirely or almost entirely inefficient for all the insects tested (Table 2 (3) and Fraenkel & Blewett [1943*a*]). In the present work the specificity of the sterol requirements of these insects is investigated.

EXPERIMENTAL

A convenient method of preparing a sterol-deficient diet is Soxhlet extraction of wholemeal flour with chloroform for at least 10 hr. Extraction with ether (Soxhlet) proved unsuccessful in removing the sterols sufficiently. Addition of various sterols fully restored the nutritional qualities of the flour. This effect was used as a means of comparing the nutritional values of sterols and sterol derivatives. In addition to the insects enumerated by Fraenkel & Blewett [1943*b*, p. 686], the flour moth *Ephestia kuehniella* Zell. was also used. The methods of experimenting and assessing results are fully described elsewhere [Fraenkel & Blewett, 1943 *a*, *c*].

The following sterols and sterol derivatives were used:

Cholesterol, sitosterol, ergosterol, calciferol, 7-ketocholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate, 7-dehydrocholesteryl monobenzoate, 7-dehydrocholesterol, cholestanol free from cholesterol (Glaxo Laboratories Ltd.).

Cholesterol purified via the dibromide, cholestanol (dihydrocholesterol), zymosterol, Δ^5-cholestene, cholesteryl acetate (Prof. I. M. Heilbron and Dr E. R. H. Jones).

RESULTS

On wholemeal flour extracted with chloroform, *Sitodrepa* and *Ephestia* did not grow at all, *Ptinus* grew very slowly with only very few insects completing development, while *Tribolium*, *Lasioderma* and *Silvanus* grew somewhat better but still at a greatly reduced rate. Adding a few drops of the chloroform extract to extracted flour restored it to almost its original efficiency. Wheat-germ oil (Vitamins Ltd.) restored extracted flour to its full nutritional value for *Tribolium* and *Ephestia* and was more efficient than the chloroform extract (Table 1 and Figs. 1, 2).

Table 1. *Growth on flour extracted with chloroform and with the addition of the extracted material or a number of sterols or sterol derivatives*

	Growth						
	With basal diet of $CHCl_3$-extracted wholemeal flour, of						With basal diet of yeast and fructose, of
Addition to basal diet	*Tribolium*	*Lasioderma*	*Silvanus*	*Ptinus*	*Sitodrepa*	*Ephestia*	*Dermestes*
None	+	+	+ +	±	−	−	−
Chloroform extract	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ + +	−
Wheat-germ oil	+ + + +	.	.	.	.	+ + + + +	.
Cholesterol	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
Sitosterol	+ + + +	+ + + +	+ + + +	+ + +	+ + + +	+ + +	−
Ergosterol	+ + + +	+ + + +	+ + +	+ + + +	+ + + +	+ +	−
Cholestanol	+ + (+)	+ +	+ + +	+ +	+ + +	+	−
Δ^5-Cholestene	+ (+)	.	.	.	−	−	−
Zymosterol	+ (+)	+ +	+ + +	+ (+)	−	−	−
Calciferol	−	±	+ +	−	−	−	−
7-Dehydrocholesterol	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
Cholesteryl-acetate	+ + + +	.	.	.	+ + + +	+ + +	+ + + +
7-Hydroxycholesterol	±	.	.	.	−	−	−
7-Dehydrocholesteryl monobenzoate	+ +	.	.	.	−	−	+ + + +
7-Hydroxycholesteryl dibenzoate	±	.	.	.	−	−	−
7-Ketocholesteryl acetate	−	.	.	.	−	−	−

The + and − signs give a rough quantitative estimate of the relative efficiency of diets. Explanation of the symbols in Fraenkel & Blewett [1943*a*].

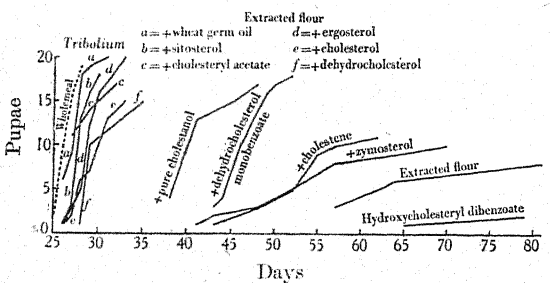

Fig. 1.

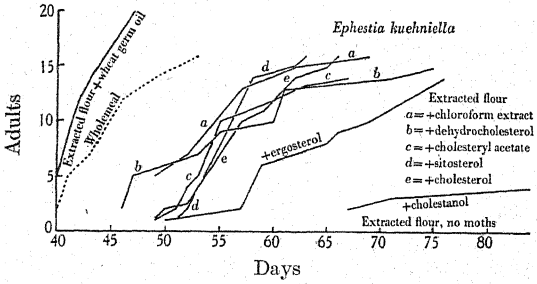

Fig. 2.

Fig. 1. *Tribolium confusum*. Growth on wholemeal flour extracted with chloroform, and on extracted flour with the addition of wheat-germ oil, sterols or sterol derivatives.

Fig. 2. *Ephestia kuehniella*. Explanation as for Fig. 1.

The results of adding sterols and sterol derivatives to extracted flour are summarized in Table 1, and are given for *Tribolium* and *Ephestia* in Figs. 1 and 2. Adding cholesterol, ergosterol or sitosterol restored its original efficiency either entirely or almost entirely. Only with *Ephestia* wheat-germ oil proved more efficient than any single sterol (Fig. 2) and from these results, and others not yet published, it seems likely that other fat-soluble factors, possibly unsaturated fatty acids, are required by *Ephestia*. Destruction of such unknown factors during the prolonged chloroform extraction at a high temperature may explain why wheat-germ oil proved more efficient for *Ephestia* than the chloroform extract. We do not yet know whether small differences in the efficiency of cholesterol, ergosterol or sitosterol are significant. Of the other sterols tested, only 7-dehydrocholesterol and cholesterylacetate proved of about the same nutritional value as the three common natural sterols.

In evaluating the effect of sterols and sterol derivatives it has to be borne in mind that extracted flour proved entirely inefficient only for *Sitodrepa* and *Ephestia*, while some growth took place with the other species. This growth was almost certainly only possible through traces of sterols remaining in the flour after extraction. Furthermore, most of the derivatives of cholesterol which we tested were not pure and almost certainly contained cholesterol, while zymosterol was probably not free from ergosterol. Traces of any physiologically active sterol would, therefore, be expected to improve further the extracted flour for those insects which already showed some growth on it by virtue of small amounts of sterol retained. The insects which required large amounts of sterol, and which entirely failed to grow on extracted flour, viz. *Ephestia* and *Sitodrepa*, grew well only on cholesterol, sitosterol, ergosterol, cholesteryl acetate and 7-dehydrocholesterol, and, to a lesser degree, on cholestanol (Fig. 2). From this it appeared probable that the seemingly positive effects of zymosterol on *Lasioderma* and *Silvanus*, and of Δ^5-cholestene, dehydrocholesterol monobenzoate and zymosterol on *Tribolium*, were due to impurities. This does not apply to cholestanol, of which a pure sample was obtained on which even *Sitodrepa* and *Ephestia* grew fairly well. Cholestanol is, therefore, of some food value, although inferior to the 'good' sterols. Calciferol (vitamin D_2) was not utilized by any of the insects tested.

DISCUSSION

It has been known for several years that sterols are growth factors for certain insects (literature in Fraenkel, Reid & Blewett [1941]) and this is now shown to be true for six more insects. It is also now certain that the fat-soluble factor necessary for *Ephestia kuehniella*, described by Richardson [1926] as vitamin A, is in fact a sterol. The last column of Table 1 contains the results of a previous investigation of the sterol requirements of another beetle, *Dermestes vulpinus* [Fraenkel *et al.* 1941]. The striking difference in the sterol requirements of *Dermestes* and the flour insects is that *Dermestes* will not grow on ergosterol or sitosterol. This limits its distribution to food of animal origin, while the flour insects grow equally well on a diet of vegetable origin (containing sitosterol), animal origin (containing cholesterol) or micro-organisms (containing ergosterol) [see Fraenkel *et al.* 1941; Fraenkel & Blewett, 1943*c*].

The present work confirms the observation that certain insects require sterols in the diet, and not calciferol (vitamin D_2), in which the tetracyclic arrangement of the carbon atoms has been changed and which is consequently not a true sterol. 7-Dehydrosterols are utilized as well as sterols with only one double bond in ring B (cholesterol, sitosterol). There is, however, no reason to assume that cholesterol and sitosterol are utilized by virtue of traces of the respective dehydrosterols which are usually associated with them in biological material. Cholesterol, purified via the dibromide and free of dehydrocholesterol, proved just as efficient as unpurified cholesterol. Of the cholesterol derivatives, only the acetic ester retained full activity, and dihydrocholesterol (cholestanol) appeared of some food value. All the other structural changes, such as loss of the secondary alcohol group in Δ^5-cholestene, introduction of a second alcohol group in 7-hydroxycholesterol, and esterification of this group in 7-hydroxycholesteryl dibenzoate or introduction of a keto-group on carbon atom 7 in 7-ketocholesteryl-acetate, caused loss of nutritional value.

Table 2. *Growth on diets slightly* (1) *or grossly* (2 *and* 3) *deficient in sterols*

Diet	Growth of *Tribolium*	*Lasioderma*	*Silvanus*	*Ptinus*	*Sitodrepa*	*Ephestia*
(1) Casein, starch, 5% yeast	+++	+++	+++	++	±	−
(2) Flour extracted with chloroform	+	+	++	±	−	−
(3) Casein, glucose, yeast extract	+	+	+	−	−	−

The requirements for sterols seem, therefore, to be qualitatively the same for the six insect species under investigation, but there are obvious quantitative differences. From a comparison of the growth on three diets relatively deficient in sterols (summarized in Table 2) it can be concluded that *Sitodrepa*, *Ptinus* and *Ephestia kuehniella* require larger quantities of sterols than *Tribolium*, *Lasioderma* and *Silvanus*.

SUMMARY

The sterol requirements of six different insect species have been investigated; all the insects require a sterol in the diet for growth. The quantitative requirements of sterols by different insects are different, but the qualitative requirements of the insects under investigation are the same. The specificity of the sterol requirements was studied.

We gratefully acknowledge a Government grant from the Royal Society, which, partly, made this investigation possible. We are also much indebted to Prof. I. M. Heilbron and Dr E. R. H. Jones (Imperial College of Science and Technology) and to Mr A. L. Bacharach and Dr E. Lester Smith (Glaxo Laboratories Ltd.) for the gift of a number of sterols.

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Apparent Vitamin C in Foods

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Titration with 2:6-dichlorophenolindophenol is used to estimate vitamin C in a wide variety of foodstuffs, though the dye may react with substances besides ascorbic acid. Harris [1933] showed that dye-reducing substances can be produced by heating certain sugars with alkali. Johnson [1933] found with germinated peas that dye titrations indicated higher vitamin C contents than was shown by biological tests. In acid extracts part of the dye-reducing power was due to a substance which reduced the dye more slowly than ascorbic acid. Bacharach, Cook & Smith [1934] found an increase in the dye-reducing power of malt extract heated to 100° for ½–2 hr. McHenry & Graham [1935] obtained increases of 50–170% by heating certain vegetables for a few minutes at 58° or by standing these for a few hours at room temperature in 1% HCl. Ahmad [1935] found that boiling cabbage for 10 min. with 20% CCl_3COOH increased the apparent vitamin C content by nearly 200%. Reedman & McHenry [1938] found that the apparent vitamin C in heated potatoes resembled true vitamin C in both physiological and many chemical properties. Their suggestion that ascorbic acid might exist in combination with protein from which it was set free by heating was discredited by Harris & Olliver [1942], who suggested three possible causes for the results: (1) incomplete extraction from unheated tissues, (2) variations in sampling, (3) inhibition by heat of the ascorbic acid oxidase in the foods examined. The possible occurrence of non-specific dye-reducing substances in vegetable tissues was considered of little or no practical importance, although it was admitted that germinated seedlings, malt extract, yeast and fermented juices might repay further study.

During the last three years we have been accumulating evidence to show that the apparent vitamin C content of foods can become considerably higher than the true vitamin C value under manufacturing and storage conditions which may occur in practice. Preliminary reference to these findings was made by Wokes, Johnson, Duncan, Organ & Jacoby [1942] and by Wokes, Organ, Duncan & Jacoby [1943*a*]. The provisional term 'apparent vitamin C' was proposed to describe substances occurring in natural and processed foods which, although not possessing antiscorbutic activity, simulate vitamin C very closely in their chemical and physical properties, so that they are not distinguished from it by the indophenol titration as usually applied, and may therefore cause results to be too high. Methods of estimating true vitamin C in the presence of this apparent vitamin C have

been described by Mapson [1943*a*,*b*] and by Wokes, Organ & Jacoby [1943*b*]. In this paper a brief account is given of the production and occurrence of apparent vitamin C in foodstuffs, and of some of its properties.

EXPERIMENTAL

Indophenol titrations

In our earlier experiments we used only the visual dye titration method essentially as described by Harris & Olliver [1942]. Later we also used the potentiometric method of Harris, Mapson & Wang [1942] with modifications. This proved invaluable both for deeply coloured products and for following dye reactions more precisely than is possible visually, and we are greatly indebted to these workers for details of the method prior to publication. Satisfactory agreement was obtained between visual and potentiometric results, provided that timing and rate of dye additions were carefully controlled as described by Wokes *et al.* [1943*b*].

Estimation of apparent vitamin C

This was carried out by a modification [Wokes *et al.* 1943*b*] of the method of Lugg [1942]. Lugg divided the various dye-reducing substances into three groups according to their rate of reaction with formaldehyde: (*a*) react readily at pH 1·5 and 3·5, (*b*) react readily at pH 3·5, slowly at pH 1·5, (*c*) do not react readily at either pH. Ascorbic acid was the only substance he had found in group (*b*); reductone occurred in group (*c*). Hence a basis was provided for differentiating ascorbic acid from reductone.

It has been shown by Mapson [1943*b*] and ourselves [Wokes *et al.* 1943*b*] that reductone and other components of apparent vitamin C react too rapidly with formaldehyde at pH 3·5 for satisfactory differentiation. We have obtained satisfactory results by treating mixtures of true and apparent vitamin C with formaldehyde at pH 4·5 for 6 min. This destroys the whole of the true vitamin C but little, if any, of the apparent vitamin C. Recovery experiments are made to correct for any apparent vitamin C destroyed in certain materials. The action of the formaldehyde is checked at the end of 6 min. by adding excess HPO_3 to bring the pH to about 1·0. To calculate true vitamin C the apparent vitamin C thus determined is subtracted from the total vitamin C estimated with similar timing and rate of dye additions.

Production of apparent vitamin C by heating with mineral acid

Potatoes, cabbages, carrots, germinated grains (wheat, barley and oats), grass and lucerne were treated with various concentrations of HCl, H_2SO_4, HPO_3 or CCl_3COOH in a water-bath at 90–95°, or occasionally on a sand-bath, water being added at intervals to make good any loss. With some materials (e.g. grass), heating on a sand-bath proved more effective. With HCl or H_2SO_4, 2% (v/v) of the concentrated acid gave the best results, producing as much apparent vitamin C as higher (up to 8%) concentrations of acid, but when less than 2% of concentrated acid was used the rate of production of apparent vitamin C fell rapidly with decreasing acid concentration. 1% HPO_3 was more effective than lower concentrations, but the rate of production of apparent vitamin C was still much slower than with 2% conc. HCl. The apparent vitamin C produced with 1% HPO_3 or with 0·25% conc. HCl was unstable to prolonged heating. When heating cabbage extract in 25% CCl_3COOH we were not able to obtain any increase in the dye titration values such as Ahmad [1935] described, but, on the contrary, as heating continued the titration value became less. Our cabbage extracts were prepared by grinding with quartz sand, with precautions to avoid destruction of ascorbic acid by oxidizing enzymes [vide Wokes & Organ, 1943]. Hence our findings indicate that the increase in the dye titration value observed by Ahmad when heating cabbage with 25% CCl_3COOH was due to extraction difficulties and not to production of apparent vitamin C. We were able to find only slight traces of apparent vitamin C in our cabbage extracts when examining these by the method of Wokes *et al.* [1943*b*]. On the other hand, when we heated germinated grains, grass and lucerne with acid, the dye titration figures steadily increased during several hours' heating (in contrast with the increase in 10 min. observed by Ahmad). The presence of apparent vitamin C was confirmed by the above method, although the amount found was sometimes less than the increase observed, and the possibility of formation of hydroxytetronic acid was not precluded. Nevertheless, ample evidence was obtained that the increase in the dye titration values was not due to any of the three possible causes mentioned by Harris & Olliver, viz. incomplete extraction, variation in sampling, or inhibition of ascorbic acid oxidase. A few representative results are given in Fig. 1.

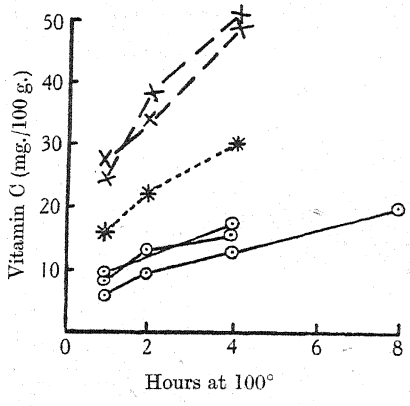


Fig. 1. Production of apparent vitamin C by heating with 2% conc. HCl or of conc. H_2SO_4 at 100°. Germinated grains, ⊙——⊙. Grass, ×——×. Lucerne, ✳·····✳.

Production of apparent vitamin C by heating without acid

In certain foods and food materials apparent vitamin C can be produced by heating to about 100° without addition of acid. This effect is strongly marked with malt extract, in which it is possible to increase the apparent vitamin C content as much as 10 times, but may also be observed with fruit syrups and other products. If true vitamin C be present, its destruction by heat may be masked by the simultaneous production of apparent vitamin C. This production of apparent vitamin C can take place at pH 4–5 as compared with a pH below 1 in the experiments with acid. A few typical results are given in Table 1.

Table 1. *Apparent and true vitamin C contents of foods, etc.*

Material	pH of material	Vitamin C content (mg./100 g.) Total	Apparent (*a*)	True	True as percentage of total
Fresh fruit and fruit preparations:					
Black currants, fresh	3·2	202	4	198	99
Black currant juice	3·2	140	5	135	96
Black currant syrup, freshly made	—	103	2	101	98
Black currant syrup after 8 weeks at room temp. in dark	—	96	4	92	96
Black currant syrup after 13 weeks at 27° in dark	—	58	3	55	94
Black currant syrup after 10 weeks at 37° in dark	—	68	8	60	88
Rose hip syrup after 12 months at 0–5°	3·7	156	20	136	88
Rose hip syrup after 12 months	3·6	101	69	32	31
Cherry juice, conc., after 5 years at 0–5°	3·8	47	45	2	4
Lemon juice, conc., after 18 months at 0–5°	1·7	193	42	151	78
Lemon juice, conc., after 4 years at 0–5°	1·7	27	21	6	22
Walnuts, unripe (*Juglans regia*), 2–3 g.	—	2619	1310	1309	50
Walnuts, unripe, 6–7 g.	5·2	2961	987	1974	67
Walnuts, unripe, 15–20 g.	3·9	2028	408	1620	80
Walnuts, unripe, after precipitation of tannin (*b*)	3·9	1909	400	1509	79
Dried fruits and vegetables:					
Dried bananas (*c*)	5·0	6	1	5	83
Dried carrots (*c*)	4·8	90	49	41	45
Dried potatoes (*d*)	4·5	27	4	23	85
Dried rose hip extract (mean of five samples) (*e*)	4·1	1300	50	1250	96
Dried spinach (*c*)	7·0	2	0·5	1·5	75
Dried tomatoes (*c*)	4·7	89	55	34	38
Malt extracts:					
Liquid malt extract, low diastatic power (D.P.)	4·7	3	(*f*)	0	0
Liquid malt extract, normal D.P.	4·7	12	(*f*)	0	0
Liquid malt extract, normal D.P.	4·8	5	(*f*)	0	0
Liquid malt extract after ½ hr. at 100°	4·8	12	(*f*)	0	0
Liquid malt extract after 2 hr. at 100°	4·8	20	(*f*)	0	0
Liquid malt extract after 9 months at 37°	3·6	103	(*f*)	0	0
Dried malt extract, low D.P. (*g*)	4·7	3	(*f*)	0	0
Dried malt extract, normal D.P. (*g*)	4·7	12	(*f*)	0	0
Sugars and molasses:					
Sugar, white (mean of three samples)	—	0	0	0	0
Sugar, brown (mean of four samples)	—	1	1	0	0
Golden syrup	—	27	27	0	0
Molasses, beet	—	18	18	0	0
Molasses, cane	—	11	11	0	0
Molasses, before charcoal treatment (*h*)	5·6	46	46	0	0
Molasses, after charcoal treatment	6·2	19	19	0	0
Molasses, beet concentrated preparation	5·4	222	(*d*)	0	0
Molasses, beet concentrated preparation	—	304	(*d*)	0	0
Molasses, cane concentrated preparation	—	196	(*d*)	0	0
Cocoa, chocolate:					
Cocoa, defatted, mixed sample	7·0	65	65	0	0
Cocoa, defatted, after precipitation of tannin	7·0	12	(*d*)	0	0
Chocolate, vitaminized	6·1	44	8	36	82
Chocolate, vitaminized	5·7	36	4	32	89
Miscellaneous:					
Beer, light draught	4·6	0·3	0·3	0	0
Beer, dark draught	4·4	1	1	0	0
Parsley (*i*)	7·0	220	40	180	82

Notes

(*a*) Apparent vitamin C determined by method of Wokes *et al.* [1943*b*] checked occasionally by Mapson's method [1943*b*].
(*b*) By method of Mirimanoff & Mori [1940].
(*c*) After about 4 years' storage in air-filled containers at room temperature.
(*d*) After 6 weeks' storage in air-filled containers at room temperature.
(*e*) pH range 4·0–4·2.
(*f*) Some of the dye reductant in these preparations reacted with HCHO under the given conditions, but since it had been produced by heating was considered not to be ascorbic acid.
(*g*) Prepared by evaporation *in vacuo* of the corresponding liquid malt extract.
(*h*) Treatment normally used in refining sugar.
(*i*) During extraction cyanide used to inhibit action of oxidizing enzymes and stabilize apparent and true vitamin C.

Development of apparent vitamin C during storage

Apparent vitamin C may be gradually formed during storage under normal conditions. The rate of formation is affected by temperature, moisture content and exposure to air and enzymic action.

(*a*) *Effect of storage temperature.* Fig. 2 summarizes some results obtained on malt extract, which show that the rate of formation of apparent vitamin C is affected by the

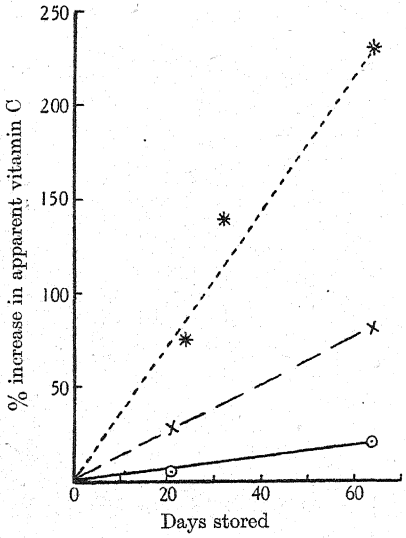


Fig. 2. Effect of storage temperature on rate of production of apparent vitamin C in malt extract. Increase at 37°, ✳-----✳. Increase at room temperature, ×——×. Increase at 0–5°, ⊙——⊙.

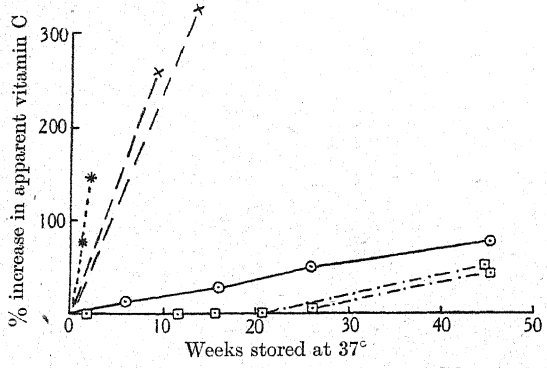


Fig. 3. Rate of production of apparent vitamin C in malt extract stored in air-filled containers at 37°, showing effect of enzyme and moisture content. Results on dried extract of low diastatic power (12 Lintner), ⊡–.–.–⊡. Dried extract of normal diastatic power (112 Lintner), ⊙——⊙. Liquid extract from which low D.P. dry extract prepared, ×——×. Liquid extract from which normal d.p. dried ext. prepared, ✳-----✳.

storage temperature. At 37° increases of 12–103 mg./100 g. were observed during 268 days' storage. In the refrigerator at about 5° an increase of 46% was observed in 150 days.

(*b*) *Effect of moisture content.* Removal of all but 1–2% of the moisture greatly retards the rate of production of apparent vitamin C. This is clearly shown by results of experiments on malt extract before and after drying. Rather less conclusive evidence was obtained, indicating that destruction of the malt enzymes also diminished the rate of production of apparent vitamin C (see Fig. 3).

In dried extracts of vegetables and fruits stored for 4 years at room temperature in air-filled containers there was marked development of apparent vitamin C, masking

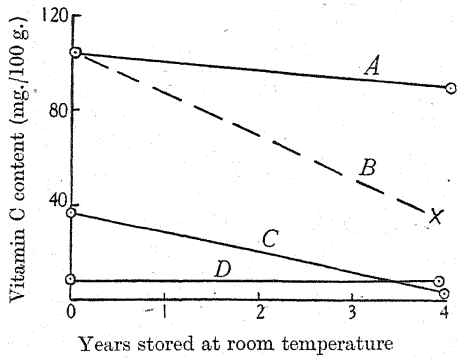


Fig. 4. Total and apparent vitamin C in dried fruit and vegetables stored in air at room temperature. *A*, total vitamin C in dried tomatoes. *B*, true vitamin C in dried tomatoes. *C*, total and true vitamin C in dried spinach. *D*, total vitamin C in dried bananas.

the gradual destruction of true vitamin C (see results with tomatoes in Fig. 4). Storage in nitrogen-filled containers reduced the rate of formation of apparent vitamin C but did not entirely prevent it.

Occurrence of apparent vitamin C in miscellaneous materials

(*a*) *Cocoa and chocolate.* Apparent vitamin C has been found in considerable amounts in cocoa, and may therefore occur in chocolate used as a carrier of the vitamin (Table 1). When the true vitamin C gradually disappears during storage, the apparent vitamin C remains, and if no correction is made for its presence the error increases in magnitude. The apparent vitamin C is not entirely tannin, since it is not completely precipitated by the method of Mirimanoff & Mori [1940].

(*b*) *Fruits and fruit syrups.* Fresh fruits appear on the whole to contain only small amounts of apparent vitamin C, which does not usually exceed 10% of the total vitamin C found. Unripe walnuts are an exception; in some species the apparent vitamin C may be 80% of the total [Melville, Wokes & Organ, 1943].

When fruit juices are heated or subjected to prolonged storage more apparent vitamin C may be produced, so that it may eventually form the greater part of the total vitamin C found by the usual dye titration (Table 1). Some of this may be reductic acid formed by the action of the fruit acids on pectin or other constituents.

(*c*) *Sugar, syrup and molasses.* Demerara and other coloured sugars contain traces of apparent vitamin C, which may produce an appreciable effect on the total vitamin C content of fruit syrups. Some samples of table syrup contain more apparent vitamin C. This is also found in molasses, from which very concentrated preparations

can be obtained. In the process of sugar refining the apparent vitamin C is presumably produced by the treatment with mineral acid, and partially removed by subsequent treatment with charcoal.

(*d*) *Vegetables.* Significant amounts of apparent vitamin C have not been found in common raw vegetables, but may be produced by cooking at a high temperature (e.g. roasting or frying), and also occur in some herbs such as sorrel and parsley.

(*e*) *Beer.* Traces of apparent vitamin C, comparable with the total vitamin C contents of South African beers reported by Levy & Fox [1935], have been found in English draught beer, especially in the dark variety.

Properties of apparent vitamin C

(*a*) *Temperature coefficient.* The rate of production of apparent vitamin C increases with rise in temperature. Between 0 and 37° the temperature coefficient for 10° rise is of the order of 2. At 100° a sharp rise in the rate occurs.

(*b*) *Effect of* pH *on rate of formation and stability.* Apparent vitamin C develops as rapidly at pH 4–5 as at pH 1, and in part at least is unstable in 5% HPO_3 solution (pH below 1) as well as in less acid aqueous solutions. Its decomposition may be retarded by bubbling nitrogen through the solution to expel air, and by storage in the dark in nitrogen-filled containers; also by the use of cyanide.

(*c*) *Effect of oxidizing enzymes.* These, including ascorbic acid oxidase, were extracted from outer cabbage leaves by the method of Szent-Györgyi [1931], from cauliflower inner florets by the method of Hopkins & Morgan [1936] and from unripe tomato skins by the method of Wokes & Organ [1943], and tested on apparent vitamin C obtained either at a pH of about 1 (e.g. beet and cane molasses, acid-heated extracts of various foods) or at a pH of about 5 (e.g. dehydrated foods, malt extract). Freshly prepared solutions in buffers of pH 2–6 were mixed with the enzyme extracts at room temperature and apparent vitamin C determined at intervals. The enzymes destroyed apparent vitamin C under the same conditions as they destroyed real vitamin C.

(*d*) *Effect of oxidizing agents.* Practically the whole of the apparent vitamin C in different foods can be destroyed within 2–3 min. by treatment with 0·1% hydrogen peroxide or sodium percarbonate at 37°. This treatment also destroys true vitamin C. With lower concentrations less of the true or apparent vitamin C was destroyed. Even $1:10^8$ dilution of H_2O_2 produced an appreciable effect. The rate of destruction was most rapid in the first minute. It did not appear possible to differentiate between true and apparent vitamin C by such means.

(*e*) *Effect of ultra-violet light.* Griffiths [1943] suggested that reductones might be formed by the action of ultra-violet light on substances in the atmosphere. Solutions of dehydrated foods, in which it was known that apparent vitamin C could be developed during prolonged storage, were irradiated (mercury vapour lamp) at pH 4–6 in covered Petri dishes for several hours, screens being used to cut out short wave-length rays not present in sunlight, without development of apparent vitamin C. Solutions of foods in which the apparent vitamin C was already present were irradiated under similar conditions, and the apparent vitamin C was gradually destroyed, having almost entirely disappeared after 3–4 hr. irradiation [Wokes, 1943]. Whilst ultra-violet light did not produce any apparent vitamin C in these dehydrated foods, the position may be quite different in natural foods, such as fruits, which are exposed to sunshine and contain various pigments and enzyme systems.

(*f*) *Spectroscopic evidence.* Dr R. A. Morton kindly examined spectroscopically three of our most concentrated sources of apparent vitamin C obtained from malt extract by prolonged incubation, and from beet and cane molasses respectively. The treatment which these had undergone precluded the presence of true vitamin C, and the absence of antiscorbutic properties was also shown by physiological tests. The incubated malt extract showed a very distinct maximum at about 280 mμ (Fig. 5). Maxima in the beet

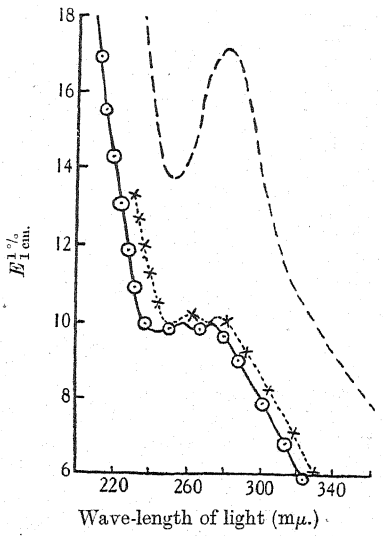


Fig. 5. Absorption spectrograms of concentrated preparations of apparent vitamin C obtained from: malt extract, ——; beet molasses, ×-----×; cane molasses, ⊙——⊙.

molasses at about 276 and 264 mμ, and in the cane molasses at about 275 and 256 mμ, were obscured by general absorption, due to the dark colour. When this dark colour was removed by treatment with charcoal, the apparent vitamin C was not recovered. Attempts to obtain colourless extracts suitable for spectroscopic examination by shaking or Soxhlet extraction with methanol or ethanol or by prolonged shaking with ether proved unsuccessful.

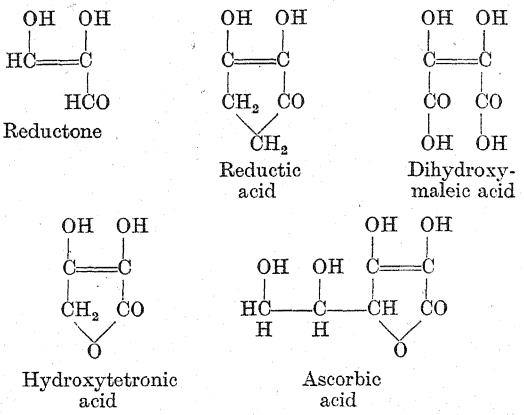
DISCUSSION

The production of non-specific dye reductants by heating different foodstuffs with or without acid has been mentioned by a number of previous workers. Very little was known of the nature and properties of these reductants and they were generally assumed not to affect the vitamin C value of foods as determined by dye titrations, either because they were not present in significant amounts or because they reacted more slowly with the dye so that their interference was obviated by carrying out the titration very rapidly, the end-point being reached in a

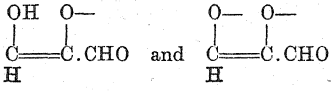
minute or less. However, some doubt has been cast on this assumption by the recent findings of Hochberg, Melnick & Oser [1943] and Taylor [1943] that, when the end-point is reached still more rapidly by extrapolation of photoelectric data, the results may be appreciably lower than those given by the ordinary visual methods. Taylor [1943] found the total vitamin C in unripe walnuts to be about 13% less by the more rapid method, and similar discrepancies occurred with other foods.

Wokes *et al.* [1943*b*], using a potentiometric method, have shown that the vitamin C value obtained for samples of dehydrated carrots, in which much apparent vitamin C had developed during 4 years' storage, was increased as longer intervals were allowed between the dye additions, thus allowing more time for the apparent vitamin C to react. With additions every 15 sec. the result was about 40 mg./100 g.; with additions every 8 min. the result was over 100 mg./100 g. It was suggested that this was due in part to enol-keto tautomerism. Apparent vitamin C was thought to exist in the solutions undergoing titration as a mixture of two isomers. Only one of these reacts with the dye. As the dye is added it removes the active isomer, and more of this is formed from the inactive isomer to restore the equilibrium. Thus the time effect is due to the rate of enol-keto tautomerism as well as to the rate of reaction of the active form with the dye.

In the present investigation it has been found that apparent vitamin C can be formed under much less drastic conditions, and occurs more widely and in greater proportions than was previously supposed. Moreover, its components resemble true vitamin C closely (1) in their susceptibility to oxidation by very low concentrations of inorganic or organic peroxides—an oxidation which is accelerated by enzyme extracts containing ascorbic acid oxidase, (2) in their removal by activated charcoal and general behaviour towards adsorbents, and (3) in their reduction of iodine, ammoniacal silver nitrate or alkaline copper solutions (Benedict's, Fehling's) in the cold. Some of the reductants in apparent vitamin C are gradually destroyed on standing in HPO_3 solution at room temperature, but may be stabilized by nitrogen or cyanide; others are stable in HPO_3 solution. They also differ from vitamin C in possessing no antiscorbutic activity and in being more stable towards heat and towards formaldehyde at pH 4–5 [Wokes *et al.* 1943*b*]. They should resemble ascorbic acid closely in chemical constitution and contain the active dienol and carbonyl groups. These are present in reductone, reductic acid, dihydroxymaleic acid and hydroxytetronic acid, four compounds closely related to ascorbic acid, as shown by comparison of their formulae.



Reductone is formed, amongst other products, when carbohydrates are treated with alkali. Such treatment may produce a variety of enolic compounds, hence the 'reductones' which Mapson prepared may have contained other dye reductants besides pure reductone, especially as their properties differed in certain respects (e.g. stability towards HPO_3) from those of our apparent vitamin C. Martius & Euler [1934] showed that reductone and dihydroxymaleic acid react much more slowly with the dye when the pH is > 5, and suggested that reductone could dissociate into two tautomeric ionized forms



Snow & Zilva [1938], when describing their experiments with ascorbic acid oxidase on reductone, suggested that their findings might be explained by the high dye-reducing power of one of these isomers, the concentration of which would be controlled by the velocity of the tautomeric conversion. A similar explanation is suggested for the variations we have observed in the reaction velocity of the dye with apparent vitamin C in different foodstuffs. Reductone has an absorption maximum at 287 mμ above pH 5·7 and at 268 mμ at pH 4·0 or lower, indicating a change in the proportion of the two isomers in this pH range which may be related to the difference in properties of apparent vitamin C in foods of pH above or below this range. The maximum at 268 mμ would be in good agreement with the maximum of 264 mμ found in our beet molasses at a similar pH, but the evidence for the presence of reductone is not conclusive. Euler & Martius [1933] stated that reductone has no antiscorbutic activity.

Reductic acid was obtained by Reichstein & Oppenauer [1933] by heating beet sugar pectin or various carbohydrate derivatives with mineral acid, treatment like that which produced apparent vitamin C in our molasses. Reductic acid has an ab-

sorption maximum at 280mμ at pH 6 and at 264mμ at pH 3 [Mayer, 1937]. The spectroscopic data on our materials do not preclude the presence of reductic acid, but the fact that the apparent vitamin C was not completely destroyed on standing in HPO_3 [Wokes *et al.* 1943*b*] suggested that the latter contained at least two different substances.

Dihydroxymaleic acid is formed when tartaric acid is oxidized by H_2O_2 in presence of small quantities of ferrous salts in sunlight. Szent-Györgyi [1939] states that it is quite common in plants, where it probably serves as a hydrogen transmitter analogous to ascorbic acid, being reversibly oxidized by an oxidase similar to ascorbic acid oxidase. Dihydroxymaleic acid, like ascorbic acid, is rapidly oxidized by traces of peroxides, but unlike ascorbic acid is unstable in acid solutions, in which it resembles part of the apparent vitamin C in our materials. Solutions of dihydroxymaleic acid in HPO_3 are not stabilized by nitrogen or cyanide. Such stabilization might be effected in the presence of certain food extracts, but our experiments in this direction have so far proved unsuccessful. Potentiometric studies of its rate of reaction with the dye confirm Szent-Györgyi's suggestion that in aqueous solution it is subject to enol-keto tautomerism. The maximum light-absorption for dihydroxymaleic acid in water is at 290mμ. This differs appreciably from the maximum at 280mμ in our incubated malt extract, and the maximum at 275–276mμ in the molasses, but the possibility must be considered that the acid in the molasses may have shifted the dihydroxymaleic acid maximum from 290 mμ to a lower wave-length. The presence of dihydroxymaleic acid in our materials is therefore not excluded. The destruction of the non-specific reductant in these materials by our enzyme extracts may have been due to their containing dihydroxymaleic acid oxidase, which Banga & Philipot [1938] found in many plants. Dihydroxymaleic acid is not antiscorbutic [Dalmer, 1934].

Hydroxytetronic acid is chemically of interest as being the parent substance from which ascorbic acid is probably formed. It resembles ascorbic acid closely in its rate of reaction with HCHO and with the indophenol dye. Its maximum absorption at 246mμ is appreciably lower than the nearest maximum of 256mμ in any of our materials, and would presumably tend to become still lower in presence of acid. Its occurrence in our materials seems less likely than that of reductic or dihydroxymaleic acids. Hydroxytetronic acid is not antiscorbutic [Micheel & Jung, 1934].

Other possible constituents of apparent vitamin C. It appears from spectroscopic evidence, and from the effects of HPO_3 and HCHO at different pH [Wokes *et al.* 1943*b*], that apparent vitamin C frequently contains more than one non-specific reductant. In addition to the reductants mentioned above, enolic substances may be present which reduce the dye fairly rapidly but which are less closely related to ascorbic acid. Certain German workers [cf. Enders, 1938; Luers, 1937] have described the occurrence and gradual development, in malted barley extracts and beer during storage, of dye reductants which they state are formed by condensation of sugars and amino-acids with evolution of CO_2. These reductants are stated not to reduce Fehling's solution, which indicates that their chemical nature differs more widely from that of ascorbic acid than does that of the above four reductants.

SUMMARY

1. The provisional term 'apparent vitamin C' is proposed to describe a group of substances occurring in foods and closely resembling ascorbic acid in chemical and physical properties (as well as in behaviour towards oxidizing enzymes), so that they are not distinguished from vitamin C by the dye titration generally employed.

2. These substances probably all contain the dienol group, and may include reductone, reductic acid, dihydroxymaleic acid and hydroxytetronic acid. They undergo enol-keto tautomerism, which causes their rate of reaction with the dye, and other properties, to be affected by the pH, the range 4–6 appearing to be critical.

3. They differ from ascorbic acid in being produced by the action of heat on certain constituents (e.g. pectins, carbohydrates) in foods and also (except hydroxytetronic acid) in their rate of reaction with formaldehyde under given conditions [Wokes *et al.* 1943*b*]. These differences in behaviour have formed the basis of methods of estimating apparent vitamin C in foodstuffs.

4. Apparent vitamin C has been found in germinated grains, malt extract, cocoa, chocolate, dehydrated fruits and vegetables, beer, and in certain fruits and herbs such as walnuts, parsley and sorrel. Particularly high contents have been found in unripe walnuts, in concentrated preparations from beet and cane molasses, and in fruit juices and dried foods stored for several years under normal conditions.

The apparent vitamin C content of processed and dehydrated foods may gradually increase during storage under normal conditions, and thus obscure loss of true vitamin C in storage experiments.

We are indebted to Dr L. J. Harris and his colleagues for advice and criticism, to Dr R. A. Morton for spectroscopic examination of various samples, to Mr H. C. Powers, chief chemist, Messrs Tate and Lyle's Thames Refinery, for samples of molasses, to Messrs W. J. Bush and Co. for samples of fruit juices and syrups, and to Dr F. Bergel for specimens of reductic and hydroxytetronic acid.

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The Osmotic Pressure of 'Defatted' Human Serum

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McCarthy showed [1938] that foetal sheep serum exerts a significantly higher osmotic pressure per gram of protein than does the maternal serum. This phenomenon could not be explained by the different albumin-globulin ratios of the two sera. Molecular weight determinations by McCarthy [1942] did not reveal any striking difference between foetal and maternal crystalline horse-serum albumins. Assuming that maternal and foetal serum albumins in the sheep are also identical, as is probable in the horse, we sought another explanation for the phenomenon observed in the foetal sheep serum. Since the lipid content of foetal blood is considerably less than that of the maternal blood [data for human: Slemons & Stander, 1923], and since Macheboeuf [1929], Macheboeuf & Sandor [1931] have stated that the lipid constituents of serum protein exert a considerable influence on osmotic pressure, it seemed desirable to investigate further the effect of lipids on the osmotic pressure of the serum proteins. Experiments were therefore undertaken to compare the osmotic pressure of serum before and after lipid extraction. The chief problem regarding the extraction was to find a suitable method which would remove the lipids without denaturing the serum proteins.

The method of McFarlane [1942] seemed the most convenient for our purpose, the method being also of especial interest since it is used in preparing pooled sera for transfusion. The method consists of shaking the serum with ether and freezing the mixture by cooling it to below $-25°$, then thawing it. The ethereal layer which separates during thawing contains a large proportion of serum lipids, and by repeated extractions approximately three-quarters of the lipids can be removed. McFarlane showed by electrophoretic measurements that the only change that takes place in the serum proteins is the diminution of the β-globulins.

METHODS

We extracted fresh pooled human serum by McFarlane's method. The serum was shaken with ether, frozen and thawed five times. Since the method employed by us may

differ slightly in detail from that of McFarlane, a summary of our procedure is desirable. The serum was frozen by surrounding the container with solid carbon dioxide ('Dricold', Imperial Chemical Industries Ltd.) and leaving it for several hours or overnight. The frozen serum was thawed at room temperature by inverting the vessel and letting the thawed liquid drip into another container. It was noticed that as the thawed serum collected, several layers separated out: usually the bottom part was crystal clear and the rest increasingly milky towards the top. After the yellow ethereal layer had been siphoned off and the extracted serum stirred up, the serum lost its opacity and became crystal clear throughout after each extraction, so that there was no need to centrifuge or filter it. While the frozen serum was thawing, a thick, creamy, yellowish material gradually separated on the surface of the ice. This remained in the first vessel; it had a low solubility in water and was not further analyzed. This material may be identical with the gelatinous deposit of low solubility in water, which McFarlane found to separate, after freezing and thawing the serum, subsequently to the last ether extraction, and which had to be filtered or centrifuged off to clear the serum. After the last ether extract had been siphoned off, the ether remaining dissolved in the serum was removed by passing nitrogen gas through the serum for approximately 1 hr. at 0°. After this, samples of the extracted serum and of the untreated serum, the latter having been kept frozen up to this time, were placed in collodion membranes and dialyzed for 48 hr. at 0° against M/15 phosphate buffer (M/30 $Na_2HPO_4.12H_2O$ + M/30 KH_2PO_4) of pH 6·8. Ten osmometers were set up at 0° with various dilutions of each sample of serum according to the method of Adair [1925], with the same buffer for the outer fluid as in dialysis. The protein concentration of the serum in each osmometer was measured by a micro-Kjeldahl determination. Albumin N was determined on three of the more concentrated sera after precipitation of the globulins by saturation with $MgSO_4$.

In calculation of protein concentration from N content, since the N contents of human-serum albumin and globulin are not known, the figures for horse-serum albumin (15·6% N) and horse-serum globulin (15·13% N) [Adair & Robinson, 1930*a*] were used. No allowance has been made for the presence of phospholipid N in the normal serum, nor for the loss of this after the extraction, since the corrections that might have been introduced are insignificant in comparison with the probable error in the determinations of total N. The serum was analyzed for its lipid content before and after the extraction. The distribution of lipids in the ether extract, and the N content of the latter, were also determined. Total fatty acids, fatty acids derived from neutral fats and cholesteryl esters, and total phospholipids were all determined by the methods of Bloor [1928, 1929]. Total free and ester cholesterol were estimated by the digitonin method as described by Popják [1943].

RESULTS

Table 1 shows the lipid content of the serum before and after extraction. It can be seen that practically all of the cholesterol, approximately two-thirds of the phospholipids and two-thirds of the neutral fats have been removed by the extraction. Table 2 shows the observed percentage distribution of lipids in the dry ether extract, together with the theoretical distribution calculated from the results of lipid analyses of the sera, assuming that all the lipids removed by the extraction were to be recovered in the ether extract. It is evident that the observed and theoretical lipid distributions differ considerably; the results appear to indicate that a large proportion of the phospholipids, neutral fats, and also of free cholesterol was lost with the material which separated on the surface of the ice during each thawing.

Table 1. *Lipid content of serum before and after extraction*

	Before extraction (mg./100 ml.)	After extraction (mg./100 ml.)
Cholesterol: Free	44	—
Ester	148	—
Total	192	Traces
Total fatty acids	390	101
Fatty acids*	280	61
Neutral fats†	183	64
Total phospholipids	171	61
Total lipids	652	125

* Fatty acids derived from neutral fats and cholesteryl esters.

† Neutral fats = (fatty acids − ester cholesterol × 0·72) × 1·05.

Table 2. *Observed and theoretical percentage distribution of lipids in ether extract of serum*

	Lipid content of ether extract	
	Observed (%)	Theoretical (%)
Phospholipid	3·20	20·90
Neutral fat	4·20	22·60
Cholesterol: Free	2·00	8·35
Ester	56·00	28·05
Fatty acids of cholesteryl esters	36·00	20·10
Total	101·40	100·00

The N content of the ether extract was found to be 0·28 mg./100 mg. of dry extract; about 22% of this small amount of N is accounted for by the phospholipids. If the remaining fraction of N is protein-N, the amount of protein in the extract must have been negligibly small.

Total N determinations on the dialyzed normal and 'defatted' sera showed a discrepancy in total protein concentration of a magnitude difficult to reconcile with any observed volume changes due to dilution in the dialysis. The total protein concentration of the dialyzed normal serum was 6·82% and that of the dialyzed 'defatted' serum 5·49% (cf. Table 3, osmometers 1 and 11 respectively). It seems probable that N determination and lipid analysis on the material which separated out on the surface of the ice during thawing would have explained the discrepancies.

The percentage albumin and globulin contents of the dialyzed normal serum were 56·76 and 43·24 % respectively; and those of the dialyzed extracted serum 61·64 and 38·36 % respectively, indicating a diminution of globulin content. It is of interest to note that a loss of β-globulin following the ether extraction of the serum was reported by McFarlane on the basis of electrophoretic analysis, although no quantitative data are available in his paper.

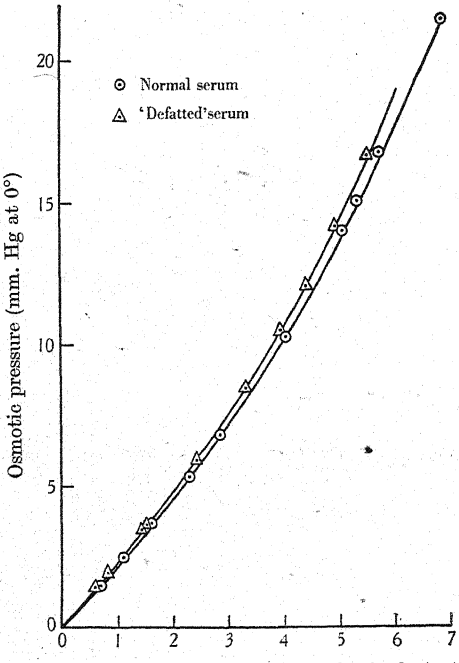

Fig. 1. Relationship between protein concentration and osmotic pressure of normal and of 'defatted' serum. The data for the two curves have been calculated from the equation of Adair: $p = \pi_0 C/(1 - K_b C)$ [cf. Adair & Robinson, 1930*b*]. K_b (normal serum) = 0·0509; K_b' (defatted serum) = 0·0547. The circles and triangles represent observed values.

Fig. 1 illustrates the osmotic pressures, in mm. of Hg at 0°, of the normal and of the extracted sera; the detailed data are presented in Table 3. It would appear that the 'defatted' serum exerts slightly higher osmotic pressures for the same protein concentrations than the normal serum. The difference in higher concentrations amounts to 0·5–0·6 mm. of Hg, in lower concentrations to 0·2–0·3 mm. of Hg. Evidence on the question whether this difference is due to the removal of lipids, or to the higher percentage of albumin in the extracted serum, can be obtained by finding the value of π_0 for both sera, i.e. the limiting value of π, i.e. the ratio

$$\frac{p \text{ (osmotic pressure)}}{C \text{ (protein concentration)}}$$

Table 3. *Data for osmotic pressures of normal and 'defatted' sera*

Osmometer no.	Osmotic pressure (mm. Hg at 0°)	Protein concentration (g./100 ml. solution)	$1/\pi$
	(*a*) Normal serum		
1	21·39	6·82	0·319
2	16·77	5·69	0·339
3	15·05	5·28	0·351
4	13·97	5·01	0·359
5	10·22	3·99	0·391
6	6·77	2·82	0·417
7	5·30	2·28	0·431
8	3·64	1·57	0·431
9	2·48	1·11	0·448
10	1·44	0·68	0·472
	(*b*) 'Defatted' serum		
11	16·66	5·49	0·329
12	14·20	4·91	0·346
13	12·11	4·35	0·360
14	10·49	3·91	0·373
15	8·50	3·28	0·386
16	5·86	2·41	0·411
17	3·63	1·51	0·417
18	3·47	1·45	0·418
19	1·95	0·82	0·420
20	1·39	0·62	0·448

in an infinitely dilute solution, and then comparing the observed and theoretical values of π_0. In the

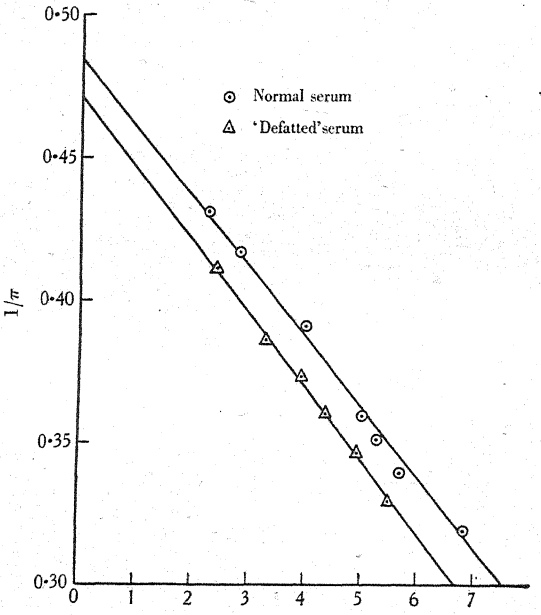

Fig. 2. Relationship between protein concentration and $1/\pi$ ($= C/p$).

comparison of osmotic pressures, it should be noted that the unequal distribution of ions may affect

results for the more concentrated solutions. This complication is eliminated by calculation of the value of π_0 for infinite dilution [Adair & Robinson, 1930*b*]. In Fig. 2 observed values of $1/\pi$ are plotted against protein concentration. The straight lines have been drawn visually as the best fit for values of $1/\pi$ relating to protein concentrations above 2 g./100 ml. of solution, and the values of $1/\pi_0$ determined by extrapolation of these lines to the ordinate. The points relating to lower protein concentrations are neglected, as the unavoidable experimental errors at these concentrations produce significant deviations in the value of $1/\pi$. The values of $1/\pi_0$ for the normal and extracted sera thus determined by extrapolation are 0·4835 and 0·4720 respectively [cf. Adair & Robinson, 1930*b*], and hence π_0 for the normal serum is 2·07, and for the extracted serum 2·12. Adair & Robinson [1930*b*] showed that for an infinitely dilute solution of serum the osmotic pressure can be expressed by the formula

$$p = RT\,[10C_a]/M_a + RT\,[10C_g]/M_g,$$

where C_a and C_g denote the concentrations (g./100 ml. of solution) of albumin and globulin, and M_a and M_g the molecular weights of albumin and globulin. In calculating the theoretical π_0, it is assumed that $M_a = 72{,}000$ and $M_g = 170{,}000$, values known for horse-serum albumin and globulin [Adair & Robinson, 1930*b*]. Then the value of theoretical π_0 is calculated by the formula

$$\pi_0 = 2{\cdot}36X_a + 1{\cdot}00\,(1 - X_a),$$

where X_a = g. of albumin/g. of total protein. For the normal serum $X_a = 0{\cdot}568$ and for the extracted serum $X_a = 0{\cdot}616$. Therefore the theoretical values of π_0 for the normal and extracted sera are 1·77 and 1·84 respectively. Thus there is an increase in the value of the theoretical π_0 of the same order as in the observed values, which is attributable to the increased percentage of albumin in the extracted serum. The difference between the values of determined and theoretical π_0 for human serum proteins may be explained by the fact that constants for horse-serum proteins were used in the computation of theoretical π_0.

Therefore we conclude from our experiment (*a*) that McFarlane's method for extracting lipids from sera removes also a part of the globulin complex without altering the colloid osmotic pressure properties of the remaining serum proteins, and (*b*) that the lipids extracted by the method do not influence the osmotic pressure of the serum proteins. The higher osmotic pressure effect of foetal serum remains still unexplained.

SUMMARY

1. Fresh pooled human serum was 'defatted' by McFarlane's method.

2. Lipid analysis of serum before and after extraction showed that practically all of the cholesterol, approximately two-thirds of the phospholipids, and two-thirds of the neutral fats, were removed by the extraction.

3. The extracted serum exerts slightly higher osmotic pressure per g. of protein than the normal serum. This difference, however, can be explained by the increased albumin-globulin ratio in the extracted serum.

4. The serum lipids, which can be removed by McFarlane's method, do not influence the osmotic pressure of the serum proteins.

We are ind ebted to Mr G. S. Adair, F.R.S., for his helpful criticism of our results and to Prof. F. G. Young for kindly allowing us to use the cold room of his department. One of us (E. F. M.) wishes to express his indebtedness to Prof. W. G. Barnard for hospitality and facilities in his department.

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The Estimation of 'True Ascorbic Acid' in Blood

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Recent work has suggested that the blood ascorbic acid concentration may provide a satisfactory index of the nutritional level of vitamin C. The concentration in whole blood is preferable for this purpose to the plasma concentration, which shows greater fluctuation; but the chief disadvantage of the blood ascorbic acid concentration as a nutritional index is the technical difficulty of estimating it accurately. The normal values obtained by different sets of workers show wide variation, and there is considerable doubt as to how much of the reducing substances estimated by these methods represents true ascorbic acid. For this reason Butler & Cushman [1940] suggest the use of the term 'apparent ascorbic acid' for the blood estimates, since they conclude that 'satisfactory criteria for the validity of whole blood analyses are lacking'.

In the present investigation the simple indophenol titration method of Deeny, Murdock & Rogan [1942] was chosen for special study. Preliminary experiments showed that a significant quantity of non-ascorbic acid reducing substance could be removed by treating the blood filtrates with lead acetate and so obtaining a precipitate of lead phosphate. This procedure removes certain SH derivatives, but not ascorbic acid [Richter & Croft, 1943]. Blood ascorbic acid figures obtained by this lead precipitation method were then compared with figures obtained on the same samples by the ascorbic oxidase method. Good agreement between estimates obtained by these two distinct methods is considered to be satisfactory evidence of the validity of the blood ascorbic acid determination.

The lead precipitation method described is very simple, and as it requires no special apparatus it appears very suitable for use in clinical investigation.

METHODS

Venous blood containing sodium oxalate (0·30 g./100 ml.) or sodium citrate (0·05 ml. saturated solution to 5 ml. blood) was used. Special care was taken to avoid haemolysis.

Lead precipitation procedure. The oxalated blood (4 ml.) was put in a 15 ml. centrifuge tube containing 0·05 ml. *sec.*-octanol, and coal gas was bubbled through. Distilled water (4 ml.) was added and the gassing continued for 10 min. 2 ml. of freshly prepared 32 % HPO_3 were added, followed after mixing by 1 ml. 25 % $Pb(Ac)_2.3H_2O$ and 2 ml. saturated sodium acetate solution. After thorough mixing, the suspension was centrifuged. Any cloudiness in the supernatant fluid indicated that the HPO_3 solution was not sufficiently fresh. 2 ml. portions of the supernatant solution were taken for titration.

The dye solution (2 mg. 2:6-dichlorophenolindophenol in 100 ml. warm water) was standardized by titration against a freshly made solution containing 2 mg. ascorbic acid in 400 ml. 2 % HPO_3. On one occasion it was found that erroneous results were due to a rapid fading of the dye solution (30 % in 24 hr.). This was traced to an impurity in the particular specimen of dye. Other specimens supplied by the same firm gave satisfactorily stable solutions, but the stability of the dye solutions should be checked before use.

The dye solution was added from a 2 ml. microburette to 2·0 ml. ascorbic acid solution in a small conical vessel made from the end of a conical centrifuge tube. The solution was stirred with a glass rod after each addition and the titration was continued until a faint pink colour persisted for 10 sec. and matched a solution made by adding 0·05 ml. of dye to 1 ml. of 1 % HPO_3 in a similar conical vessel: the amount of dye solution added in the blank was subtracted from the final burette reading. As the pink colour at the end-point was very faint, it was essential that the titration should be done against a white background and in a good light. After some practice, duplicate estimations showed good agreement.

In calculation of the result a small correction was applied for the ascorbic acid contained in the protein precipitate obtained in deproteinization. The precipitate was found to contain 65 % of water, by drying to constant weight. With the volumes of blood and reagents given the total volume was 13 ml., of which about 2·2 ml. were occupied by the moist precipitate. The effective volume of the aqueous solution was therefore taken as 12·2 ml. The ascorbic acid concentration in whole blood (mg./100 ml.) was obtained by multiplying the mg. ascorbic acid found in 2 ml. of the supernatant solution by the factor $\frac{1}{2}(12{\cdot}2) \times \frac{1}{4}(100) = 152{\cdot}5$.

RESULTS

Removal of non-ascorbic acid reducing substances with lead acetate. Ascorbic acid estimations were carried out on a series of blood specimens by the method of Deeny *et al.* [1942]. It was then found that when the blood filtrates obtained after deproteinization with HPO_3 and sodium acetate were treated with 1 ml. 25% lead acetate solution, and the lead phosphate precipitate was removed by centrifuging, titration with the indophenol dye generally gave a significantly lower result, owing to the removal of non-ascorbic acid reducing substances (Table 1). Allowance has been made for the dilution by the lead acetate solution.

Table 1. *Ascorbic acid content of fresh and of stored blood*

Time of storage (days)	Total dye reducing substance, estimated as ascorbic acid (mg./100 ml.)	'True ascorbic acid' by lead precipitation (mg./100 ml.)	Non-ascorbic acid reducing substance (%)
	(1) Oxalated blood		
0	0·78	0·70	11
0	0·60	0·54	11
	(2) Citrated blood		
0	0·58	0·54	7
2	0·57	0·43	33
6	0·63	0·57	10
6	0·46	0·38	21
6	0·46	0·38	21
6	0·25	0·23	9
6	0·67	0·50	34

The stored specimens were kept at 3°.

Estimation by ascorbic acid oxidase. That the lower ascorbic acid figures obtained after lead acetate treatment were due to the removal of non-ascorbic acid reducing substances was confirmed by estimating the ascorbic acid by an independent method. The ascorbic oxidase was used for the quantitative oxidation of the ascorbic acid. The oxidase was prepared from cauliflower and the experimental procedure was that of Richter & Croft [1943]. The use of this method was made difficult by the relatively low concentration of ascorbic acid in the blood filtrates and for this reason the method cannot be recommended for blood; but with great care satisfactory agreement of duplicates was obtained. Each figure given in Table 2 represents the means of two separate estimations, i.e. of six burette readings.

It was clear that the figures obtained by the ascorbic oxidase method agreed very much more closely with those obtained by the lead precipitation method procedure (correlation coefficient 1·00) than with the figures for the total reducing substance given by the method of Deeny *et al.* [1942] (correlation coefficient 0·96). The closeness of the agreement gave evidence of the reliability of the lead precipitation method.

Table 2. *Estimation of ascorbic acid in blood by (a) ascorbic acid oxidase and by (b) lead methods*

Total reducing substance as ascorbic acid (mg./100 ml.)	'True ascorbic acid' (a) By oxidase method (mg./100 ml.)	'True ascorbic acid' (b) By lead method (mg./100 ml.)
	(1) Oxalated blood	
0·50	0·37	0·36
0·36	0·31	0·30
0·22	0·21	0·21
0·62	0·56	0·56
	(2) Citrated blood	
0·51	0·42	0·44
0·31	0·27	0·26
0·40	0·30	0·32

The correlation coefficient between the total reducing substance and the figures obtained by the ascorbic oxidase method was 0·96: between figures by the lead precipitation method and the ascorbic oxidase method, 1·00. The blood specimens were used within 3 hr. of collection.

Subsequent work showed that it was unnecessary to carry out the two precipitations separately and to precipitate with lead acetate after a preliminary deproteinization: identical figures were obtained when these two stages were combined as described under 'Methods'.

The stability of ascorbic acid in citrated whole blood

Heinemann [1938] and Kassan & Roe [1940] reported that the level of ascorbic acid in whole blood remained constant for 16–24 hr. at room temperature and for 52 hr. at 0°. As it was desired to analyze specimens sent by rail and by post, this point was further investigated. Estimations were made on specimens of citrated and oxalated blood immediately after collection and after storage for 24 and 48 hr. at various temperatures, with or without occasional shaking (Table 3).

There was no significant loss of ascorbic acid under these conditions for 24 hr., and even in 48 hr. the maximum loss observed in 10 specimens was only 22%. The range of blood ascorbic acid concentrations is wide—from about 1·5 mg./100 ml. in the fully saturated individual to below 0·15 mg./100 ml. in a person with scurvy. In clinical work only the approximate level is usually required in order to demonstrate or to exclude the existence of gross deficiency. A loss of even 22% would not render the result entirely useless for this purpose.

Table 3. *Stability of ascorbic acid in citrated and oxalated whole blood*

Anti-coagulant	Percentage of initial ascorbic acid remaining after (hr. of storage) 0	24	48	Initial ascorbic acid content (mg./100 ml.)	Temp. (°)
Saturated citrate	100	100	—	0·30	16
	100	96	—	0·27	16
	100	98	91	0·55	14
	100	100	84	0·48	14
3·8% citrate	100	100	80	0·27	21
	100	—	100	0·19	20
	100	—	90	0·15	20
	100	—	78	0·30	20
	100	—	84	0·26	20
	100	97	91	0·32	19
Oxalate	100	97	85	0·34	20
	100	100	89	0·30	20

Blood preserved with saturated citrate contained 0·05 ml. saturated sodium citrate solution to 5 ml. blood; with 3·8% citrate, 2·5 ml. 3·8% sodium citrate solution to 10 ml. blood; with oxalate, 0·03 g. sodium oxalate to 10 ml. blood.

DISCUSSION

In attempts to assess the nutritional level of vitamin C in a subject, either the saturation test [Harris, 1942] or the blood ascorbic acid concentration can give useful information. The saturation test is simple to carry out, but its usefulness is limited in that it requires the accurate collection of urine, dosage with ascorbic acid and repeated estimations for a period of several days. The blood ascorbic acid concentration, on the other hand, is so constant that a single estimation is usually adequate, and it has the great advantage that the estimation need not be carried out immediately after collection of the blood specimens. Ascorbic acid is stabilized by the reducing systems present in citrated or oxalated whole blood, so that there is no significant loss on storage for 24 hr. at ordinary temperature. Estimations can thus be carried out satisfactorily even on specimens sent by post. The chief disadvantage of the blood concentration as a nutritional index is the difficulty of estimating it accurately. There is, however, no standard accepted method for the estimation of blood ascorbic acid, and some of the methods in current use are subject to serious criticism [Butler & Cushman, 1940]. Several photoelectric absorptiometric and other methods have been described which have given good results in the hands of experienced research workers, but they are too difficult for routine use by the average clinical laboratory technician and we must agree with Bessey [1938] that their accuracy has generally been overrated.

It is evident from the present investigation that the blood ascorbic acid figures obtained by methods such as that of Deeny *et al.* [1942] are too high, owing to the inclusion of a variable amount of extraneous reducing substances. The lead precipitation method described corrects this source of error and gives somewhat lower figures. In order to obtain the greatest possible accuracy a relatively large volume of blood (4 ml.) was used and the volume of the other additions was kept small. After some practice in recognition of the end-point of the titration, duplicate estimations showed very close agreement, and the reliability of the method was confirmed by comparison with the ascorbic oxidase method for estimating 'true ascorbic acid'.

The method has been applied satisfactorily to estimation of the blood ascorbic acid in factory workers in the Birmingham area: the blood specimens were sent by rail and titrated within 24 hr. at Mill Hill. The blood ascorbic acid concentration of 350 normal individuals varied from 0·15 to 0·85 mg./100 ml., with a mean level of 0·25 during the months of May, June and July 1943. Fruit and vegetables were scarce in the Birmingham area during this period.

SUMMARY

1. Figures obtained for the blood ascorbic acid concentration by certain current methods are too high owing to the inclusion of a variable amount of extraneous reducing substance.

2. A simple lead precipitation method is described which eliminates this source of error.

3. The validity of the estimates by the lead precipitation method was demonstrated by comparison with the ascorbic oxidase method for 'true ascorbic acid'.

4. There is no significant loss of ascorbic acid in specimens of citrated whole blood kept for 24 hr. at 20°.

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The Estimation of Inositol in Animal Tissues

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Inositol occurs in animal tissues in both free and combined forms. The occurrence of water-soluble combined inositol was first suggested by Rosenberger [1908*a*,*b*, 1910]. Comparing the inositol contents of fresh rabbit muscle and commercial beef muscle, he came to the conclusion that animal tissues contain 'inositogen'. This may be identical with the 'tyrophenosit' of Danileffski [1884], who reported, without giving data, a considerable increase in the inositol content of mice after 6 days' autolysis at 37° in the presence of $CHCl_3$. Needham [1923] found that the skeletal muscle of a freshly killed rabbit contained only one-half the amount of inositol found in that of a rabbit killed 2 days previous to the estimation. An increase in the free inositol content of the skeletal muscle of rats occurred after autolysis of the tissues [Needham, 1924]. Winter [1934], working on cardiac muscle of dogs, postulated the occurrence of three forms of inositol in animal tissues: 'free' inositol, determined after ½ hr. boiling with 10% KOH; 'combined' inositol, determined after 5 hr. boiling with 10% KOH, and 'newly formed' inositol, liberated after incubation at 37° in nitrogen. The cardiac muscle of the sheep, ox and pig contained no 'combined' inositol [Winter, 1940]. It is doubtful, however, whether much significance can be attached to any of Winter's results, owing to the unreliability of the chemical methods he employed. Woolley [1941*a*] obtained inositol after acid or alkaline hydrolysis of an ethanol-insoluble, H_2O-soluble, non-dialyzable substance isolated from liver. This was considered to be probably a phosphoric acid ester of inositol. Rapoport [1940] found that in bird and turtle erythrocytes a large proportion of the organic acid-soluble P is present in the form of phytic acid. Besides being present in animal tissues in water-soluble combined forms, inositol also occurs in brain and spinal cord bound in a phosphatide fraction. Folch & Woolley [1942] isolated inositol from an acid hydrolysate of brain cephalin, and found that it constituted up to 10% of this phosphatide fraction and amounted to as much as 0·4% of the weight of the brain.

It is evident that further work is required to elucidate the nature of the water-soluble combined forms of inositol in the animal body and the methods of breakdown and significance of both water-soluble and water-insoluble combined forms. Recent developments have, in fact, followed on the application of a microbiological technique [Williams, Stout, Mitchell & McMahan, 1941; Woolley, 1942]. The chemical methods at present available for the estimation of inositol in biological material [see Needham, 1923, 1926; Winter, 1934, 1940; Young, 1934*a*,*b*; and Gregory, 1935] are, however, tedious, lengthy and involve the use of relatively large amounts of tissues. The method to be described is much quicker than previous ones and possesses several other advantages, including improvements in the extraction of inositol from tissues, the removal of interfering substances by ion exchange materials and the estimation of inositol, without isolation, by oxidation with periodic acid.

METHODS

Principle of the method of estimation

Dried tissue is extracted with water and the fractions insoluble in 70% acetone and soluble in ether both removed from the aqueous extract. Glucose is then removed by yeast fermentation and both acidic and basic substances present in the extract removed by adsorption on a mixture of ion exchange adsorbents. The free inositol present in the extract is then quantitatively oxidized with HIO_4, the excess HIO_4 being estimated iodometrically. Suitable corrections are applied for any HIO_4 used in oxidizing the glycerol which may be present in the extract at this stage.

Water-soluble combined inositol is determined after acid hydrolysis of the aqueous extract.

Experimental

Preparation of tissue for extraction. As soon as possible after death, the tissue is removed from the animal, sectioned on a freezing microtome and the sections dried in thin layers in Petri dishes *in vacuo* in the cold room over silica gel. Drying is completed in an oven at 110°, after which the material is powdered and kept in the cold until analyzed. The water content of another portion of the same tissue is determined by drying to constant weight at 110°.

Extraction of inositol. An amount of dried powdered tissue containing approximately 0·5–1·0 mg. inositol (i.e. 1·0–2·0 g. dry skeletal muscle powder) is brought to the boil with 50 ml. distilled water, filtered under reduced pressure through a small plug of cotton-wool in a funnel, and the vessel and filter washed with two portions of 5 ml. of distilled water. This process is repeated twice, the three

extracts pooled and evaporated to about 50 ml., and acetone added to give a final concentration of 70%. The precipitate is filtered off and the acetone removed from the filtrate by distillation. The extract is evaporated down to about 25 ml. and extracted with ether. The ether extract is washed once with about 20 ml. distilled water, and the washings added to the aqueous residue, which is then concentrated to approximately 30 ml. If it is desired to estimate the 'combined' water-soluble inositol as well as the 'free' inositol the aqueous extract can be divided into two portions and one hydrolyzed as described below.

Removal of glucose. The only satisfactory method for the removal of glucose was found to be yeast fermentation, as used by Young [1934*a*]. Since some products of glucose fermentation have to be removed on the ion exchange adsorbents, the fermentation stage was carried out before the adsorption.

A washed yeast suspension is prepared by shaking one part by weight of pressed baker's yeast with five parts of distilled water, and centrifuging; this process is repeated four times and the suspension of washed yeast then made up to the original volume with distilled water. The suspension is prepared fresh each day and its potency tested by incubating 2 ml. with 25 ml. glucose solution (containing 5 mg. glucose) for 10 min. at 37°, and estimating the reducing power of the supernatant fluid, obtained by centrifuging, by Hagedorn & Jensen's ferricyanide method, a blank being set up at the same time with yeast suspension and water only.

The tissue extract is incubated at 37° with 2 ml. of the yeast suspension for 10 min. and then centrifuged. The supernatant liquid is siphoned into a boiling tube and the yeast washed twice by centrifuging with 10 ml. portions of distilled water, the washing being kept separate from the main supernatant fluid for the subsequent adsorption.

Preparation of ion exchange materials and the removal of interfering substances from tissue extracts. After treatment with yeast, the extract is purified by the use of ion exchange materials, with a carbonaceous zeolite for cation exchanges and 'M.P.D. Resin' as an acid adsorbent. Both these are obtainable from Permutit Company, Limited. A brief account of the use of these substances for the purification of muscle extracts has been published [Platt & Glock, 1942]. The removal of creatine and creatinine from muscle extracts by this method proved to be much more efficient than by the precipitation technique of West & Petersen [1932] with $HgSO_4$ and $BaCO_3$, which was employed by Young [1934*b*]. Treatment of the extract with a mixture of regenerated, washed and dried carbonaceous zeolite and 'M.P.D. Resin' is more efficient than successive treatments with the two materials separately. With this technique, however, the adsorbing materials cannot be regenerated.

250 g. or more of the carbonaceous zeolite are shaken gently at intervals for a period of 1 hr. with 2 vols. of 2N HCl, filtered on a Buchner funnel and washed on the funnel with a total volume of approximately 5 l. of distilled water. The final washings should be neutral to litmus. This regenerated, washed cation exchange material is dried at 100° and sieved so that the particles are of 20/40 mesh sieve size. The 'M.P.D. Resin' is treated in a similar way, but with 2N NH_4OH instead of HCl. At least 1 l. of hot distilled water should be used to wash this reagent, in order to remove excess *m*-phenylene diamine which is liable to be present and which, if not removed completely, gives a blank reading with HIO_4. These two regenerated, washed, dried and sieved materials are mixed in equal parts by weight; this preparation will be called the 'adsorbent'.

15 g. of adsorbent are introduced into a tube approximately 20 in. by $\frac{7}{16}$ in., narrowed at the bottom to approximately $\frac{3}{16}$ in., and fitted with a small piece of rubber tubing carrying a glass tip and provided with a screw clip. The material is kept in position in the tube with a loosely packed plug of glass-wool. Before being treated with the tissue extract, the adsorbent is washed with a total volume of approximately 100 ml. of distilled water. The final washings must give no blank when allowed to react with 5 ml. 0·01M HIO_4 for 10 min. at room temperature. To prevent the formation of air bubbles in the column, which would impede the passage of liquid, the tube should contain distilled water into which the adsorbent is poured, the level of the liquid being kept above the top of the adsorbent. After the adsorbent has been washed sufficiently, the supernatant liquid from the yeast treatment is poured into the tube and allowed to drip through the column at a rate such that the whole of the liquid passes through in approximately 90 min. When the liquid has passed through completely, the first 10 ml. portion of the yeast washings is poured into the tube, and the rate of flow adjusted so that this liquid passes through in approximately 15 min. The tube containing the column of adsorbent is sucked dry on a filter pump, and then a second 10 ml. portion of yeast washings is allowed to drip through the column, which is then sucked dry. The column is washed with the help of suction with 15 successive 5 ml. portions of distilled water. This amount of washing is found to be necessary and sufficient to obtain quantitative recovery of 1 mg. of added inositol.

The assumption has been made that the only HIO_4-reacting substances which are not removed from yeast-fermented muscle extracts by the adsorbent mixture are inositol and glycerol.

Oxidation with HIO_4. At the suggestion of Prof. H. Raistrick, we investigated the possibility of estimating inositol by oxidation with HIO_4. Malaprade [1928 *a*, *b*, 1934] found that polyhydroxy alcohols were oxidized quantitatively by HIO_4 at room temperature. Fleury & Joly [1937 *a*, *b*] used periodate for the estimation of inositol both in pure solution and in the presence of glucose, but under the conditions used by them the oxidation, when allowed to go to completion, used up much more periodate than was to be expected from the equation

$$C_6H_6(OH)_6 + 6HIO_4 = 6HIO_3 + 6H.COOH,$$

and they came to the conclusion that secondary reactions occurred.

By allowing the reaction to go to completion at low temperatures (6–8°), we have found that the utilization of HIO_4 is quantitative. Formic acid, determined by titrating to pH 5·5 with 0·01N NaOH, with a mixture of chlorophenol red and bromocresol green as indicator, has been produced to the extent of about 90% of the theoretical amount by the time the utilization of HIO_4 has reached the theoretical figure. Thereafter no more formic acid is formed. Some typical results for the reaction between HIO_4 and inositol in varying concentrations at 8 and 30° are given in Fig. 1.

Differential oxidation of inositol and of glycerol by HIO_4. After the removal of glucose from muscle extracts by fermentation with yeast, small amounts of glycerol remain. Some, no doubt, is extracted from the muscle and an

amount equivalent to approximately 4% of the glucose fermented is produced by fermentation. Attempts to remove glycerol by making use of its slight solubility in ether, its reported solubility in dry ethyl acetate, and its fermentation by *Bacillus proteus*, all failed. Glycerol, in concentrations of 0·2–0·9 mg./100 ml. solution, is, however, oxidized completely and quantitatively by 2 ml. 0·01M HIO_4 at 8° in 90 min.

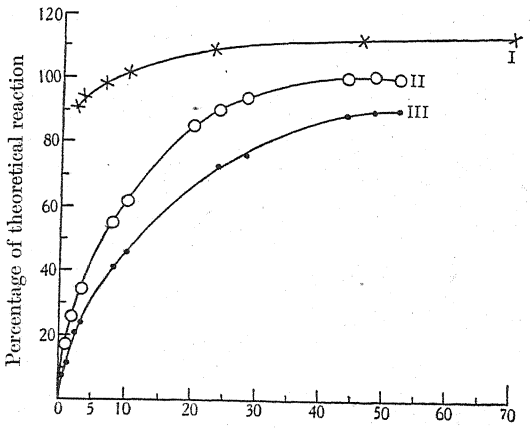

Fig. 1. Reaction of inositol with periodic acid. Extent to which the reaction

$$C_6H_6(OH)_6 + 6HIO_4 = 6H.COOH + 6HIO_3$$

is followed.

I, 1 mg. inositol/10 ml. + 5 ml. 0·01 M HIO_4 at 30°.
II, 1 mg. inositol/100 ml. + 10 ml. 0·01 M HIO_4 at 8°.
III, 1 mg. inositol/100 ml. + 5 ml. 0·01 M HIO_4 at 8°.

The percentage oxidation of glycerol (0·8 mg./100 ml.) by 2 ml. 0·01M HIO_4 at 8° at intervals up to 100 min. is as follows:

Time in min.	20	30	40	60	70	80	90	100
% oxidation	53·0	68·6	79·8	95·6	98·0	100	100	100

The time for complete oxidation of glycerol depends on the temperature at which the reaction is carried out and on the concentration of both glycerol and HIO_4: e.g. at 6° 1 mg. of glycerol in 10 ml. water is completely oxidized by 3·0 ml. 0·01 M HIO_4 in 15 min.

Concentrations of inositol of 0·5–2·5 mg./100 ml. react to the extent of 1·7–2·2% with 2·0 ml. 0·01M HIO_4 at 8° in 90 min. It was therefore necessary to determine (*a*) the total HIO_4-reacting material (inositol plus glycerol) in 100 ml. of solution treated with 10 ml. 0·01M HIO_4 at 8° for 48 hr., and (*b*) the material reacting under the same conditions of concentration and temperature with 2 ml. 0·01M HIO_4 in 90 min. (*b*) includes the whole of the glycerol and approximately 2% of the total inositol. Thus the difference between (*a*) and (*b*) multiplied by 1·02 gives the inositol content of the solution.

Absence of HIO_4-reducing substances other than glycerol and inositol from fermented effluent. There is support for the assumption, that glycerol and inositol are the only HIO_4-reacting substances left after fermentation and adsorption, in the fact that the glycerol content of the purified extracts, calculated from the HIO_4 reduced under the conditions stated above, is identical with that calculated from the formaldehyde formed, estimated as the dimedone derivative. The only other possible substances which would react with HIO_4 are amino-acids. Tests for NH_3 production (from β-hydroxy amino-acids) with HIO_4 in strongly alkaline solution in a Conway unit were always negative, as also were tests for amino-N by formol titration.

Estimation of free inositol. The effluent from the adsorption procedure is made up to 200 ml. in a graduated flask and 100 ml. withdrawn. Both portions are cooled to 8°. To one portion are added 10 ml. 0·01M HIO_4 (also at 8°) and the reaction allowed to proceed for 48 hr. To the other portion, 2 ml. HIO_4 are added and the reaction is allowed to proceed at 8° for 90 min. If a temperature other than 8° is selected the time relationships for oxidation of both glycerol and inositol at this temperature must be determined. The reaction is stopped in both cases by adjusting the pH to approximately 6·5 by the addition of 5 ml. phosphate buffer (12 g. $Na_2HPO_4.12H_2O$ and 20 ml. N H_2SO_4/100 ml.), followed by 5 ml. 5% KI solution. After 5 min. the I_2 liberated is titrated with approximately 0·004N $Na_2S_2O_3$ with 1% soluble starch in saturated NaCl as indicator, the end-point being stabilized by the addition of a few drops of saturated $NaHCO_3$ solution. 1 mg. inositol contained in 100 ml. distilled water is also allowed to react with 10 ml. 0·01M HIO_4 for 48 hr. at 8°, and the excess HIO_4 titrated as above.

The HIO_4 reduced is obtained from the difference between the titration of the purified tissue extract after reaction with HIO_4 and the blank titration obtained with the HIO_4 alone.

Let x = HIO_4 reduced in terms of 0·004N $Na_2S_2O_3$ in 48 hr. at 8°,

y = HIO_4 reduced in terms of 0·004N $Na_2S_2O_3$ in 90 min. at 8°,

z = HIO_4 reduced by 1 mg. inositol in 48 hr. at 8°.

The approximate inositol content in mg. of half the extract is given by $(x-y)/z$ which should be multiplied by 1·02 (see above).

Estimation of 'combined inositol'. For the estimation of the water-soluble combined inositol, the extract is hydrolyzed with acid [Woolley, 1941*b*]. Both water-soluble fractions (i.e. 'free' and 'combined') are determined in the same sample of tissue, with twice as much tissue as in the procedure outlined above. Before fermentation, the extract is made to 50 ml., and one-half is refluxed for 6 hr. with HCl (final concn. 18% HCl). The HCl is removed by distillation under reduced pressure, and the contents of the flask are washed out with distilled water to give a total volume of approximately 25 ml. and then neutralized by the dropwise addition of N NaOH.

Experimental diets

Albino rats were put on an experimental diet, containing only small amounts of inositol, at 6 weeks of age and were in good condition when killed after an experimental period of 16½ months. The diet consisted of casein (A/E, Glaxo), 24%; sucrose, 67·5% (replaced by maize dextrin, after 13½ months on diet, for 2 months and by rice starch for last month); McCollum's salt mixture (185), 4%; wheat-germ oil, 2%; dried yeast (*Torula utilis*, 95% dry matter, containing not more than 2·7 mg. inositol/g.), 2·5%, in-

creased to 5% after 2 months on the diet; ascorbic acid 2·5 mg. daily; vitamin A approximately 22·5 I.U. daily and vitamin D approximately 4·5 I.U. daily (both contained in 2 drops of Radiostoleum diluted with peanut oil). Between 8 and 12·5 g. of this diet, mixed to a thick paste with water, were fed daily to each rat.

RESULTS

Inositol content of rat tissues

Table 1 contains the mean results of analyses of the tissues of four rats for water-soluble inositol, free and total (i.e. including combined).

Table 1. *Inositol content of rat tissues*

	Water-soluble inositol (mg./100 g. tissue)			
	Free		Total	
Tissue	Dry wt.	Wet wt.	Dry wt.	Wet wt.
(*a*) Rats given 5 mg. inositol/day				
Skeletal muscle	49·3	12·9	81·2	21·3
Liver	85·2	24·8	178	52·1
Heart	141·3	34·3	186	45·3
Brain	241	55·8	286	67·0
Kidney	328	88·0	456	123
(*b*) Rats given no extra inositol				
Skeletal muscle	24·0	6·3	76·2	18·3
Liver	89·0	25·5	174	50·3
Heart	—	—	—	—
Brain	169	37·8	295	66·1
Kidney	316	79·5	426	113

Our values for the total water-soluble inositol contents of rat tissues are of the same order as those found by other workers, including those recently obtained by Williams *et al.* [1941], with a microbiological method, on tissue autolysates. The proportions of free to total water-soluble inositol are also of the same order as those obtained by Woolley [1942], who also used a microbiological method.

It will be seen that there is no important difference between the values for the total water-soluble inositol for the rats receiving the inositol-deficient diet, and those with added inositol. It thus appears that rats are able to synthesize inositol, which confirms the earlier work of Needham [1924]. Woolley [1942] also showed that mice could synthesize inositol in the gut if the diet was not deficient in pantothenic acid.

SUMMARY

1. A chemical method is described for the estimation of inositol in animal tissues. This possesses advantages over earlier methods in that the time taken for an estimation is considerably reduced, the method of extraction of inositol from the tissues improved, the tedious precipitation procedures replaced by treatment with ion exchange materials, and the inositol finally estimated in solution by reaction with HIO_4 without isolation of the inositol.

2. Both free and total water-soluble inositol were determined in the kidney, heart, liver, brain and skeletal muscle of control rats, and of rats receiving a daily supplement of 5 mg. inositol. No appreciable difference was found between the total water-soluble inositol contents of the tissues in the two groups of rats.

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The Determination of Ascorbic Acid in the Presence of Sulphur Dioxide

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The determination of ascorbic acid with aqueous 2:6-dichlorophenolindophenol is not entirely specific for this vitamin unless special techniques for eliminating interfering substances are applied in each case. SO_2 has a particularly strong reducing action on indophenol, and, when used for the preservation of fruit juices and cordials or in the processing of dried fruits and vegetables, should be removed before the ascorbic acid content of these materials can be accurately determined.

Three methods for eliminating free SO_2 have been described: (1) By boiling the extract and distilling off SO_2 [Kirkpatrick, 1941*a*; Downer, 1942]. (2) By passing a stream of O_2-free N_2 through the extract [Mapson, 1942]. (3) By formation of the non-reducing bisulphite compound of SO_2 with acetone [Mapson, 1942]. SO_2 in fruit juices sweetened with glucose or invert sugar forms the bisulphite compound with the hexose. In amounts of SO_2 up to 1300 parts per million (p.p.m.), indophenol titration is not affected unless the sugar concentration falls below 20% [Kirkpatrick, 1941*b*].

The first two methods are lengthy, and only the third would be practicable as a routine method when many samples are to be examined. In an attempt to find a method by which accurate results may be obtained without the necessity of a fixed rate of titration, the effect of adding H_2O_2, which rapidly converts SO_2 quantitatively into non-interfering H_2SO_4, has now been investigated. Initial experiments showed that the addition of an equal volume of 3% H_2O_2 to an ascorbic acid solution acidified with one drop of concentrated HCl had no appreciable effect on the value obtained by indophenol titration, when the dye was added from the burette. The ascorbic acid was thus unaffected by the H_2O_2, and a diminution in titration value was experienced only when Fe or Cu salts were added to the ascorbic acid, and the solution was allowed to stand for some time before titration.

EXPERIMENTAL

In a detailed study of the effect of adding SO_2 and varying quantities of H_2O_2 to ascorbic acid extracts from various sources, the experiments summarized in Table 1 were carried out. The end-point in each case was reached within 1 min.

Solutions and reagents employed

Ascorbic acid solution: 6·25 mg. dissolved in 100 ml. distilled water. For each titration 10 ml. were acidified with one drop of concentrated HCl.

Orange juice solution: 20% (v/v) in distilled water.

Cauliflower leaf: extract made with 2% HPO_3.

Parsley: extract made with 2% HPO_3.

0·05% *aqueous 2:6-dichlorophenolindophenol*: 1 ml. was equivalent to 0·25 mg. ascorbic acid.

Hydrogen peroxide: a 3% solution of B.P. quality.

$KHSO_3$ *solution*: a 0·934% (w/v) solution in distilled water. 1 ml. added to 10 ml. ascorbic acid solution produced a SO_2 concentration of 450 p.p.m.

DISCUSSION

Table 1 shows that H_2O_2 does not appreciably oxidize ascorbic acid, whether the latter is contained in pure aqueous solution (Exp. 2), orange juice (Exp. 8), or HPO_3 extract of vegetables (Exps. 16 and 19), even after standing for 6 min. The use of HPO_3 as extractant has been reported to guard against the danger of oxidation of ascorbic acid by Fe or Cu, and this probably explains why cauliflower and parsley extracts give good values after H_2O_2 addition, in spite of traces of dissolved mineral salts. The addition of more Fe or Cu salt, however, causes slow oxidation (Exp. 21). H_2O_2 has no effect on unreduced indophenol (Exp. 6).

In all experiments the addition of $KHSO_3$ gives rise to very high titration values, but H_2O_2 neutralizes the effect of the SO_2, and the original titration figure is obtained.

In all experiments in which H_2O_2 has been added to ascorbic acid solutions, the end-point with indophenol is a permanent pink. This colour deepens at varying speeds due to reoxidation of the reduced and decolorized indophenol (leuco-compound) to the original coloured form. The rate of reformation of indophenol is very slow with a concentration of 1·5–2% H_2O_2 (Exps. 10 and 11) and is more rapid with 0·075% H_2O_2 (Exp. 14). The reoxidation of reduced indophenol is still more rapidly catalyzed with added Fe and Cu salts (Exp. 21). The diminution in the titration value with lower concentrations of H_2O_2 (e.g. 0·075%) is most probably due to

Table 1. *Experiments showing the effect of H_2O_2 on the indophenol titration of sulphited and unsulphited ascorbic acid extracts*

Exp. no.	Solution used (ml.)	$KHSO_3$ added (ml.)	3% H_2O_2 added (ml.)	Indophenol titration value (ml.)	End-point and remarks (A = pink; permanent 1 min. B = pink; colour deepens very slowly on standing)
	Ascorbic acid				
1	10	—	—	2·5	A
2	10	—	10	2·45	B
3	10	1	—	>4	Strongly reducing
4	10	1	10	2·45	B
5	10	—	10 (stood 6 min.)	2·3	B
6	Nil	—	10 + 10 ml. water and 1 drop conc. HCl	<0·05	A
	Orange juice				
7	10	—	—	3·9	A
8	10	—	10	3·9	B
9	10	1	—	>7	Strongly reducing
10	10	1	20	3·8	B
11	10	1	10	3·8	B
12	10	1	5	3·8	Pink colour deepens slightly more rapidly
13	10	1	1	3·7	Colour deepens more rapidly
14	10	1	0·5	3·6	Colour deepens rapidly
	Cauliflower leaf				
15	10	—	—	1·52	A
16	10	—	10	1·52	B
17	10	1	10	1·52	B
	Parsley				
18	10	—	—	6·65	A
19	10	—	10 (stood 6 min.)	6·6	B
20	10	1	10	6·65	B
21	10	1 + traces $FeCl_3$ and $CuSO_4$	10	6·1	Pink colour deepens very rapidly

reoxidation of reduced indophenol during the titration, and reduction of the reformed indophenol by ascorbic acid still present. A great advantage of the H_2O_2 procedure is that the pink end-point does not fade.

General method for estimation of ascorbic acid in solutions containing sulphite

The acidity of a measured portion of the solution is brought below pH 3·5 by the addition of HCl, if necessary, and a volume of 3% H_2O_2 equal to that of the whole is added. Indophenol is run in from a burette until a permanent pink colour is obtained. The end-point should be reached within 1 min.

SUMMARY

1. A method for the elimination, by means of H_2O_2, of the interference of SO_2 in ascorbic acid titrations is described.

2. Under the conditions used, H_2O_2 has no appreciable effect on ascorbic acid, unless Fe or Cu salts are present.

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The Determination of Ascorbic Acid in the Presence of Interfering Substances, Notably Reductones

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Reductone, reductic acid and other compounds formed in hot alkaline sugar solutions reduce 2:6-dichlorophenolindophenol in the same way as does ascorbic acid. In fact Kertesz [1934] suggested a method of determining the ascorbic acid equivalent of indophenol solutions by titrating the gluco-

reductone produced under standard conditions from glucose and NaOH. Shinohara [1938] observed that the reaction of ascorbic acid with iodine was slow when the acidity was greater than that corresponding with N/2 HCl, the end-point being described as indefinite. This fact has now led to a more detailed investigation into the effect of strong acid on the titration of ascorbic acid with indophenol or iodine.

EXPERIMENTAL

Titration with indophenol

When concentrated HCl is added to ascorbic acid solutions, reversible oxidation becomes progressively slower as the HCl concentration increases. When the HCl content reaches 20% (2·5 parts of 36% HCl to 2 parts of solution) or more, the pink colour produced with one drop of indophenol remains visible for 2 min. On dilution of this solution with 5 vol. of water, indophenol is again reduced to the same extent as in non-acidified solution. The end-point is definite and stable.

Exp. 1. *Stability of ascorbic acid in the presence of* 20% HCl. A solution of ascorbic acid was prepared of indophenol equivalent 3·95 ml./10 ml. Portions of 20 ml. were treated with 25 ml. 36% HCl. This mixture gave no titration. At different times 100 ml. water were added to these 20 ml. portions, and the solution was titrated, the indophenol being added from the burette.

Time (hr.)	Indophenol equiv. (ml.)
0	3·95
1	3·95
3	3·95
24	3·6

The acid thus has no effect on the final titration value after dilution, in periods up to 3 hr.

Exp. 2. *Fresh orange juice titrated with and without the addition of concentrated* HCl:

2 ml. orange juice ≡ 2·3 ml. 0·05% indophenol,
2 ml. + 3·0 ml. 36% HCl ≡ 0·4 ml. indophenol.

2·5 ml. 36% HCl suffices for an initial concentration of 20% HCl, but an extra 0·5 ml. was added in order to compensate for dilution of the acid by the aqueous indophenol. The end-point was taken when the pink colour persisted for 20 sec. ('20 sec. end-point'). On dilution with 25 ml. water the titration was continued to 2·3 ml. The ascorbic acid equivalent is therefore considered to be 1·9 ml. indophenol, the residual 0·4 ml. being due to interfering substances.

Exp. 3. *Guava, lucerne and parsley.* Extracts of these, prepared with HPO_3, gave no indophenol titration in the presence of 20% HCl. These substances thus contain no interfering material, and the total titration is due to ascorbic acid. On dilution with water the full titration value is obtained.

Exp. 4. *Glucoreductone.* A solution of this was prepared by the method of Kertesz [1934]. Of this solution 2 ml. were equivalent to 1·8 ml. indophenol (5 sec. end-point). Preliminary tests showed that the mixture (2 ml. solution + 2·5 ml. 36% HCl) reduced indophenol. The titration was carried out in the following manner. As in Exp. 2, an extra 0·5 ml. HCl was first added, and then the indophenol slowly, with shaking. After the addition of each 0·4 ml. indophenol, an extra 0·5 ml. HCl was required. The end-point (persistence of pink colour for 20 sec.) was reached with 1·8 ml. indophenol and 2·5 ml. additional acid. Thus, under the conditions described, glucoreductone reduces indophenol fully in the presence of HCl. The rate of reduction of indophenol was very much retarded.

Exp. 5. *Titration of ascorbic acid in presence of reductone.* An aqueous ascorbic acid solution was prepared, 1 ml. of which was equivalent to 0·8 ml. indophenol (constant end-point):

2 ml. glucoreductone + 1 ml. ascorbic acid
≡ 2·6 ml. indophenol (5 sec. end-point),

2 ml. glucoreductone + 1 ml. ascorbic acid + HCl
(3·8 ml. giving initially 20% HCl + 5 additional quantities of 0·5 ml., as in Exp. 4)
≡ 1·8 ml. indophenol (20 sec. end-point).

On dilution of the last solution with 25 ml. water, a renewed titration of 0·8 ml. indophenol (constant end-point) resulted. The titration in the presence of strong acid gives the glucoreductone equivalent. After dilution with water the extra titration gives the ascorbic acid equivalent.

Differentiation between ascorbic acid and glucoreductone. Three separate titrations are involved: (1) At pH *c.* 3 the titre represents the two substances together. (2) In the presence of at least 20% HCl, indophenol titrates only glucoreductone. The difference between the titrations (1) and (2) represents the ascorbic acid present. (3) On dilution of the reaction mixture from (2) with water and continuation of the titration to the final end-point, the exact equivalent of ascorbic acid is reached. This final titration serves as a check on the value found above by difference.

This technique was successfully applied to an examination of biscuits which had been fortified with concentrated orange juice. High vitamin C values were claimed for these biscuits, but it was found that on titration ordinary plain biscuit gave figures of 20–60 mg. apparent ascorbic acid/100 g. These values were obviously due to reductone-like substances extracted by HPO_3. When the biscuit extracts were treated with HCl as described above, no diminution in the titration value was observed. Thus no ascorbic acid was present in either of the samples. The vitamin C from the orange juice concentrate must have been destroyed in the process of baking.

Titration with N/400 *iodine*

The titration of ascorbic acid with iodine is also affected by the addition of concentrated HCl. The addition of the mineral acid to ascorbic acid solutions at first decreases the rate of reversible oxidation. When a concentration of 29% HCl is reached, iodine is no longer reduced, a higher acid concentration being required for this stage to be reached than in the case of indophenol. On dilution with water the total ascorbic acid reducing capacity is restored. These titrations are best carried out by

adding the iodine to the solution in a 50 ml. pear-shaped separating funnel, containing 1 ml. of $CHCl_3$, and then shaking after the addition of each drop. One drop of N/400 iodine in excess produces a definite pink coloration in the $CHCl_3$ layer. Alternatively, the technique of McHenry & Graham [1935] for the titration of coloured extracts can be applied. At the very high acid concentration required, the titration of glucoreductone is no longer accurate. Consequently, iodine is not a suitable reagent for distinguishing between glucoreductone and ascorbic acid.

Estimation of ascorbic acid under various conditions

(*a*) *In the presence of* $Na_2S_2O_3$. Aqueous solutions of $Na_2S_2O_3$ give the full titration value both with indophenol and iodine in the presence of the requisite amount of HCl, although the rate of reduction is greatly diminished. Either reagent may therefore be used to determine ascorbic acid in the presence of $Na_2S_2O_3$.

(*b*) *In fermented products.* A fermented brew known as 'kaffir beer' made from maize or sorghum malt contains a small amount of ascorbic acid [Delf, 1921]. It has been shown that titration of the brew with indophenol must be carried out at pH 1–2 in order to obtain reliable results [Fox & Stone, 1938]. In the presence of excess HCl a remarkable reaction takes place in that the titration with indophenol is increased instead of decreased. With the iodine technique described above the true ascorbic acid content of the beer can be correctly determined, as in the following experiment:

(*a*) With starch as indicator, 5 ml. beer + 1 drop HCl ≡ 1·0 ml. N/400 iodine.

(*b*) 5 ml. beer + 20 ml. HCl (1 ml. chloroform) ≡ 0·85 ml. N/400 iodine. In this case 4 drops of concentrated HCl were added with each drop of indophenol to maintain the concentration of acid.

(*c*) On dilution of the last solution with water a titration of 1·0 ml. N/400 iodine was obtained.

5 ml. beer thus contained ascorbic acid equivalent to 0·15 ml. N/400 iodine, i.e. 0·66 mg. ascorbic acid/100 ml. This value agrees with that obtained by indophenol titration at pH 1·5.

(*c*) *In urine.* The addition of HCl causes a darkening of urine, and the end-point of the reaction with indophenol becomes difficult to see. This can be greatly improved by preparing a comparison flask of urine and acid. When iodine is the reagent, the end-point is readily seen in the pink colour of the $CHCl_3$ layer.

At about pH 3 the combined titration of ascorbic acid and $Na_2S_2O_3$ in urine gives a satisfactory end-point, but the titration of $Na_2S_2O_3$ in the strongly acid medium is not complete. In the presence of this substance the values found for ascorbic acid would, therefore, be too high.

A suggested explanation of the behaviour of ascorbic acid with concentrated HCl

A solution of ascorbic acid is a tautomeric mixture of the dienol- and keto-enol forms. In a weakly acid solution the mobility of the ionizable H atom permits enolization. However, in the presence of an abundance of H ions due to the addition of HCl, depression of the ionization results in the vitamin being converted completely into the keto-form. Both indophenol and iodine react with the enol-form and not at all with the keto-form. For complete titration free tautomerism from the keto- to the enol-form is necessary. Since no titration takes place with strong HCl, it is probable that only the keto-form is present. On dilution with water, enolization is again facilitated, and titration proceeds to completion.

SUMMARY

1. Ascorbic acid is inert to indophenol or iodine in presence of 20% or 29% HCl, respectively, but on dilution with water the usual reaction occurs.

2. Indophenol is reduced in presence of 20% HCl by glucoreductone and other interfering substances.

3. Ascorbic acid determination in the presence of glucoreductone by a differential titration method is described.

4. It is suggested that, in presence of moderately concentrated HCl, ascorbic acid is in the unreducing keto-form.

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Studies on the Synthesis of Hyoscyamine in *Atropa belladonna* L. and *Datura stramonium* L.

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In previous papers [Cromwell, 1933, 1937], experiments on the biosynthesis of the alkaloids berberine and hyoscyamine in *Berberis darwinii* and *Atropa belladonna* respectively led to the conclusion that these alkaloids are synthesized from products of carbohydrate and protein breakdown.

This work has been continued with the object of throwing further light on the nature of the intermediate reactions leading to the formation of hyoscyamine in *Atropa belladonna* and *Datura stramonium*.

METHODS

Material. Plants of *Atropa belladonna* and *Datura stramonium* were raised from seed and set out in plots or grown in pots.

Preparation of material for estimation of total alkaloid. Leaves were rapidly dried at a temperature of 60° in an oven and ground to fine powder. Samples of root ½ in. in diameter were detached from the crowns in the case of *Atropa*, washed thoroughly, cut into pieces and dried at 60°. Roots of *Datura* were also washed, and dried at 60°. When completely dry, the roots were ground to fine powder.

Estimation of total alkaloids. Leaf or root powder (5–10 g.) was exhaustively extracted with dry ethanol in a Soxhlet extractor. The extract was transferred to an evaporating basin and the ethanol removed on a water-bath at 60–70°. The resinous residue was extracted with 50 ml. 0·5 N H_2SO_4 and subsequently washed twice with 25 ml. portions of acid. The combined extracts and washings were next shaken with 100 ml. of a mixture of 4 vol. of ether and 1 vol. of chloroform, to remove impurities. The acid extract was made alkaline with dilute ammonia and the bases shaken out with three 100 ml. portions of the ether-chloroform mixture. The three extracts were combined and the solvent distilled off. The residue was treated with two portions of 10 ml. ether which were completely removed by evaporation at 80°. Volatile bases and free ammonia were driven off by heating on the water-bath at 100° for 15 min. The residue was dissolved in 25 ml. N/50 H_2SO_4 and the excess titrated with N/50 NaOH with methyl red as indicator, and the results calculated in terms of hyoscyamine. (1 ml. of N/50 acid≡0·005784 g. hyoscyamine.)

Recovery of hyoscyamine as picrate gave proof that this base is chiefly responsible for the titration values obtained. The theoretical amount of aqueous picric acid was added to the alkaloid solution after neutralization, and the crystals (plates) purified by recrystallizing twice from water. The picrate obtained had m.p. 163–164°. (Found: N, 10·89%. Calc. for hyoscyamine picrate, N, 10·81%.) Pure hyoscyamine picrate has m.p. 165°.

The results obtained from four plants of *Atropa belladonna* are given in the following table:

	Wt. of powder (g.)	Titration value (ml. N/50 H_2SO_4)	Titration value (as g. hyoscyamine)	Theoretical wt. of picrate (g.)	Picrate found (g.)	% recovery
(1)	5 (leaf)	3·9	0·022	0·039	0·0242	62
	5 (root)	5·8	0·033	0·059	0·0386	65
(2)	10 (leaf)	8·1	0·046	0·082	0·0597	73
	10 (root)	10·3	0·059	0·105	0·0793	75
(3)	20 (leaf)	16·7	0·096	0·172	0·1446	84
	20 (root)	20·1	0·116	0·207	0·1782	86
(4)	50 (leaf)	43·2	0·249	0·447	0·4120	92
	50 (root)	54·3	0·314	0·562	0·5152	92

RESULTS

Alkaloid and carbohydrate content of Atropa belladonna

(1) *Normal plants (seasonal variation).* Samples of root, stem and leaves were taken from 2-year-old plants. The figures given in Table 1 represent the average content of six plants. Qualitative experiments on carbohydrate distribution are recorded in Table 2.

Table 1. *Seasonal variation of hyoscyamine content of* Atropa belladonna

Month	g. alkaloid/100 g. dry wt. Root	Stem	Leaf
Jan.	0·42	—	—
Feb.	0·40	—	—
Mar.	0·38	—	0·60 (shoots)
Apr.	0·42	—	0·51 (shoots)
May	0·56	—	0·39
June	0·59	0·12	0·45
July	0·62	0·16	0·48
Aug.	0·61	0·14	0·45
Sept.	0·60	0·10	0·43
Oct.	0·61	0·10	0·43
Nov.	0·56	—	—
Dec.	0·52	—	—

(2) *Etiolated plants.* At the beginning of March, before growth commenced, large plant pots were placed over the crowns of plants. Control samples were taken of roots. At the end of May further

Table 2. *Seasonal distribution of carbohydrates*

	Summer	Autumn	Winter
Roots:			
Reducing sugars	+	+	+
Non-reducing sugars	+	+	+
Starch	+ (wood and pith)	+ + +	+ + +
Stems:			
Reducing sugars	+	+	
Non-reducing sugars	+ +	+	
Starch	–	Trace	
Leaves:			
Reducing sugars	+	Trace	
Non-reducing sugars	+ +	+	
Starch	–	Trace	

Tannins were present in leaves, stems and roots in all seasons. Arginine was present in leaves and roots.

examination of roots was made and also of the etiolated shoots which had been formed. The results of this experiment are shown in Tables 3 and 4.

Table 3. *Hyoscyamine content of etiolated plants*

	g. alkaloid/100 g. dry wt.		
	Roots		
Plant	Control	Experimental	Etiolated shoots
A	0·40	1·10	0·43
B	0·37	0·98	0·45
C	0·41	1·20	0·41
D	0·37	0·88	0·45
E	0·45	1·51	0·47
F	0·42	1·52	0·47

Table 4. *Distribution of carbohydrates in etiolated plants*

	Roots	Etiolated shoots
Reducing sugars	–	+
Non-reducing sugars	+	+ +
Starch	+	–

Arginine was present in both roots and shoots.

The translocation of hyoscyamine in Atropa belladonna

The upward and downward movement of alkaloid was studied by two methods, (*a*) examination of the exudate from cut stems and (*b*) grafting experiments.

Exudate from stems. The shoots of 2-year-old plants were cut to within 3 in. of the crown, and stout rubber tubing, provided with a screw clip, immediately slipped over the stumps. Glass tubes were inserted and supported by retort stands. 10 ml. distilled water were run into the tubes and the level marked. After one week had elapsed the exudate from the stumps was collected and estimated for hyoscyamine. 20 ml. of the exudate from each of three plants gave respectively 1·1, 1·3 and 0·9 mg. hyoscyamine.

Grafting experiments. Experiments were carried out using *A. belladonna* roots as stocks and tomato shoots as scions and vice versa. Samples of root were removed from *A. belladonna* stocks before the experiment, and hyoscyamine estimated. After a period of 5 weeks the tomato scions were examined qualitatively and quantitatively for alkaloid. The Vitali test was applied to a water extract of one of twelve scions and gave a strongly positive reaction for hyoscyamine. The remaining scions were dried and estimated quantitatively. The results are shown in Table 5.

Table 5. *Hyoscyamine content of* A. belladonna *stocks and of tomato scions*

		g. alkaloid/100 g. dry wt.	
		Control	Experimental
(*a*) *A. belladonna* stock:	A	0·52	0·51
	B	0·56	0·53
	C	0·52	0·50
	D	0·54	0·51
(*b*) Tomato scions		0·12	—

The grafting of *A. belladonna* on tomato stocks proved unsatisfactory, only two scions out of twelve surviving. The stocks carrying these scions gave a trace of hyoscyamine by the Vitali test. These experiments show that upward movement of alkaloid from the root system of *A. belladonna* takes place freely, while downward movement from the leaves is very restricted.

The influence of the injection of various substances on the production of alkaloid

Solutions of potassium nitrate, ammonium sulphate, asparagine, urea, nitrogenous bases and amino-acids, alone and in association with glucose, were injected into plants of *Atropa* (2 years old) and *Datura*, with the object of observing the effect on the plants and on the production of alkaloid.

Petiolar and stem injections were first carried out but did not prove satisfactory. Injection through the stump of a stem proved the most rapid and reliable method of introducing solutions into *Atropa* plants, and branch injection in the case of *Datura*.

The solution was contained in a litre aspirator bottle placed 3 ft. from the ground and was conveyed to the cut end of the stump through stout rubber tubing provided with a screw clip. To avoid air locks the tubing was filled with solution by opening the screw clip before connexion was made. With good transpiration as much as 400 ml. of solution passed into *Atropa* plants in 3 days. 100 ml. was the average volume which passed into *Datura* plants. Samples of leaves and roots were taken before and after the injection period.

Preliminary injections were carried out with eosin solution to determine the extent and distribution of injected fluid, and it was found that the dye reached the greater part of the root and shoot system of the plants. In these experiments 2% solutions of glucose and 0·25% of all other substances were used and the duration of injection was 7 days. None of the substances injected at these concentrations appeared to injure the plants.

Table 6. *Influence of the injection of various substances on the alkaloid content of the plants*

(Values given represent the mean of three injections on separate plants.)

Solution injected	g. alkaloid/100 g. dry wt. Leaf Control	Leaf Experimental	Root Control	Root Experimental
A. *Atropa belladonna*				
Potassium nitrate	0·37	0·36	0·55	0·56
Do. +glucose	0·45	0·47	0·64	0·63
Ammonium sulphate	0·43	0·47	0·53	0·55
Do. +glucose	0·47	0·49	0·56	0·60
Asparagine	0·43	0·44	0·60	0·62
Do. +glucose	0·42	0·46	0·59	0·62
Glycine	0·39	0·41	0·55	0·58
Do. +glucose	0·46	0·48	0·63	0·66
Arginine	0·45	0·48	0·65	0·67
Do. +glucose	0·44	0·52	0·58	0·61
Lysine	0·45	0·45	0·55	0·58
Do. +glucose	0·41	0·44	0·52	0·54
Proline	0·42	0·45	0·55	0·56
Do. +glucose	0·41	0·45	0·59	0·61
Putrescine (dihydrochloride)	0·44	0·67	0·62	0·65
Do. +glucose	0·44	0·70	0·60	0·67
Cadaverine (dihydrochloride)	0·39	0·40	0·60	0·62
Do. +glucose	0·41	0·41	0·63	0·64
Choline (chloride)	0·40	0·42	0·61	0·62
Do. +glucose	0·44	0·48	0·60	0·64
Betaine (hydrochloride)	0·45	0·45	0·69	0·65
Do. +glucose	0·39	0·41	0·61	0·62
Methylamine (hydrochloride)	0·42	0·43	0·58	0·60
Do. +glucose	0·38	0·42	0·66	0·69
Dimethylamine (hydrochloride)	0·45	0·45	0·62	0·64
Do. +glucose	0·41	0·45	0·64	0·65
Trimethylamine (hydrochloride)	0·43	0·42	0·57	0·58
Do. +glucose	0·49	0·52	0·59	0·59
Hexamethylene tetramine	0·47	0·49	0·72	0·72
Do. +glucose	0·42	0·47	0·58	0·61
Formamol (hexamethylene tetramine-anhydro-methylene citrate)	0·45	0·48	0·55	0·58
Do. +glucose	0·43	0·49	0·62	0·68
Succinimide	0·44	0·43	0·65	0·67
Do. +glucose	0·49	0·49	0·69	0·68
Urea	0·43	0·43	0·58	0·55
Do. +glucose	0·41	0·42	0·61	0·60
B. *Datura stramonium*				
Asparagine	0·31	0·34	0·25	0·26
Do. +glucose	0·32	0·36	0·26	0·29
Putrescine (dihydrochloride)	0·33	0·33	0·22	0·25
Do. +glucose	0·32	0·36	0·22	0·29
Glycine	0·29	0·31	0·27	0·30
Do. +glucose	0·33	0·36	0·25	0·29
Arginine	0·34	0·34	0·29	0·32
Do. +glucose	0·33	0·36	0·30	0·32
Formamol	0·31	0·35	0·28	0·30
Do. +glucose	0·36	0·39	0·27	0·31

The results of the injection experiments are shown in Table 6; an increase of 0·05 g. hyoscyamine/100 g. dry weight is considered significant.

Injections of arginine with glucose, putrescine alone and with glucose, hexamine with glucose and formamol with glucose gave substantial increases in the hyoscyamine content of *Atropa belladonna*. Putrescine with glucose and formamol alone gave the best results with *Datura stramonium*.

Material from a second experiment on the injection of putrescine was used to check the titration values obtained in the first experiment, and to endeavour to show, by isolation as picrate, that increase of hyoscyamine is responsible for the increased titration value after putrescine injection (Table 7).

The theoretical amount of picric acid was added to the combined neutralized solutions from three plants of *Atropa belladonna* to ensure a good recovery of the alkaloid picrate. 10 g. samples of leaf and root powder were extracted in each case.

Volatile base content of normal and etiolated plants of Atropa belladonna

The volatile bases pyridine, *N*-methyl pyrrolidine and *N*-methyl pyrroline are present in the leaves and roots of *Atropa belladonna* and *Datura stramonium*. Unfortunately a reliable method for separating and estimating these bases could not be devised, but it seemed desirable to examine in normal and etiolated plants the content of volatile bases extractable with ether-chloroform mixture. The procedure adopted was similar to that for the estimation of hyoscyamine with the exception that N NaOH was used for the liberation of the bases, which were then distilled into standard acid. The base content was calculated in terms of *N*-methyl pyrrolidine (Table 8).

DISCUSSION

As a preliminary to further investigations on the nature of the intermediates in the synthesis of hyoscyamine, it was considered desirable to obtain more information on the behaviour of the alkaloid in the plant organs throughout the entire year, and also to carry out translocation experiments with the object of locating the tissues in which synthesis takes place.

The results of experiments on the exudate from cut stems and on grafting indicate clearly that hyoscyamine moves in an upward direction from root to shoot system, and will pass into a scion which does not normally contain it. Although

Table 7. *The effect of injection of putrescine on the yield of hyoscyamine*

	Leaf		Root	
	Control	Experimental	Control	Experimental
(1) Putrescine (dihydrochloride):				
Combined titration value of 3 plants (g. hyoscyamine)	0·123	0·180	0·166	0·184
Theoretical weight of hyoscyamine picrate (g.)	0·220	0·322	0·297	0·329
Weight of picrate found (g.)	0·1871	0·2706	0·2583	0·2896
(2) Putrescine (dihydrochloride) + glucose:				
Combined titration value of 3 plants (g. hyoscyamine)	0·139	0·203	0·155	0·188
Theoretical weight of hyoscyamine picrate (g.)	0·249	0·363	0·277	0·336
Weight of picrate found (g.)	0·2138	0·3195	0·2383	0·2862

The picrates obtained had m.p. 163–164° (N content of sample 10·79 %).

these experiments do not prove that the root system is the locus of synthesis, the evidence is strongly in favour of this view. In plants growing under normal conditions it is therefore probable that the alkaloid is formed principally in the root and moves upwards through the vessels into the stems, leaves, fruits and seeds. It would appear that downward movement is very restricted. The work of Dawson [1941] on the tobacco plant similarly leads to the conclusion that nicotine is synthesized mainly in the root and that it moves from the root system via the xylem to the leaves. Shmuck, Smirnow & Ilyin [1941] also conclude from their grafting experiments that the synthesis of nicotine by *Nicotiana* is intimately connected with the root system and the stem. Kerkis & Pigulevskaya [1941] found atropine in all parts of tomato scions grafted on *Datura stramonium*.

Table 8. *Volatile base content of normal and etiolated plants*

	Content of volatile base calculated as g. *N*-methyl pyrrolidine/100 g. dry wt.	
(*a*) Normal plants		
	Leaves	Roots
Plant A	0·07	0·04
B	0·09	0·06
C	0·07	0·05
(*b*) Etiolated plants		
	Shoots	Roots
Plant D	0·12	0·09
E	0·14	0·13
F	0·18	0·10

The leaves of *Atropa* plants growing in full sun generally give a higher yield of alkaloid than plants growing in shade, and it is possible that the greater transpiration occurring in such plants would tend to draw up more alkaloid from the root system. Sievers [1921], working with *Datura* spp., found that removal of flower buds brought about an increase in alkaloid content of leaves. This finding may be interpreted on the assumption that alkaloid which would normally reach the fruits and seeds would be diverted to the leaves.

The seasonal distribution of alkaloid in roots, stems and leaves justifies, in the main, conclusions drawn from translocation experiments. For leaf tissues a minimum occurred in May (sufficient fully developed leaf material was not available before May), and a maximum in July, after which there was a slight fall until October, when the plants were killed by frost. The low concentration in the stems and the comparatively high concentration in the leaves during autumn show that little downward movement had taken place and therefore, on the subsequent death of leaves and stems, a considerable amount of alkaloid is lost to the plant. The peak of concentration is coincident with full maturity of the plant.

In normal seasons buds develop on the crowns during late autumn and remain dormant throughout the winter. Mild winters, however, may induce considerable growth of these buds, which may assume the form of shoots in February.

Analysis of entire shoots made in early March reveals a substantially high alkaloid content, but leaves detached in May from strongly growing shoots give yields which are low for leaf tissues. In the root a minimum occurs in March and a maximum in July, and from July until November the concentration is well maintained. From November to March an appreciable loss takes place which may be correlated with the development of buds and shoots at this period. From conclusions reached regarding translocation it would appear that loss of alkaloid takes place from root to shoot system. Later work, however, has shown that synthesis may take place to some extent in shoots developing in absence of light, and this was previously found

to be true for leaves from which light was excluded [Cromwell, 1937].

The distribution of carbohydrates follows the course usual for the majority of perennial plants. Starch is the principal carbohydrate stored in the roots during winter and is mobilized in the spring. During the summer months a trace of starch is present in the leaves, whereas mobile sugars are abundant in both leaves and stems. The high concentration of alkaloid found in the roots of plants from which light had been excluded during initial development is particularly significant. Non-reducing sugars and arginine also are present in roots and etiolated shoots, and therefore conditions for synthesis would be optimum, on the assumption that products of carbohydrate breakdown and amino-acids are the essential raw materials. On the other hand, it could be argued that the high content of alkaloid in the roots of these plants is the result of little translocation having taken place into the poorly developed etiolated shoots. Subsequent work has shown, moreover, that etiolated shoots from plants receiving similar treatment are able to synthesize alkaloid to a small extent. The yield of volatile bases is also stimulated by subjecting the plants to continued darkness for 3 months at the beginning of the growing season; hence it may also be assumed that conditions leading to accumulation of amino-acids and carbohydrate derivatives favour synthesis of these bases.

In the injection experiments an increase in alkaloid content of 0·05% over that of the controls is considered significant, although, if limiting factors are operating, it would not necessarily follow that values below this figure rule out the possibility that the substances in question act as intermediates in alkaloid synthesis. It is to be noted that, after injection, the increase of alkaloid in the leaves is usually more marked than in roots, but if synthesis is taking place in the root and the alkaloid moves to the leaves large variations in the root would not be expected. In many instances the stimulating effect of glucose is apparent and is probably due to the provision on breakdown of a ready source of energy and also of intermediates for hyoscyamine production.

Before discussion of the effects of specific substances on injection, it will be convenient to refer to laboratory syntheses of derivatives and near relatives of hyoscyamine. Robinson [1917] synthesized tropinone from succindialdehyde, methylamine and acetone-dicarboxylic acid, highly reactive substances which might occur in the plant. Robinson considers that succindialdehyde and methylamine may arise from the interaction of formaldehyde and ornithine, and acetone-dicarboxylic acid from citric acid. Schöpf & Lehmann [1935] showed that pH affected the yield of tropinone, and obtained at pH 7 a yield of 73% and at pH 5 90%. Schöpf [1937] synthesized teloidine from mesotartaric dialdehyde, methylamine and acetone-dicarboxylic acid.

Of the various amines and amino-acids injected into plants of *Atropa belladonna* and *Datura stramonium*, putrescine, arginine, hexamine and formamol are responsible for the greatest increase in the yield of alkaloid. The effect of putrescine, alone and in association with glucose, is especially marked and suggests that this diamine is an intermediate in the synthesis of hyoscyamine. Arginine injected in association with glucose likewise increases the content of alkaloid substantially. It is therefore inferred that arginine occupies a key position in the synthesis of hyoscyamine as the precursor of putrescine. In processes of bacterial putrefaction, putrescine is generally regarded as arising from arginine via ornithine, and in this connexion Gale [1940] has shown that a mixture of washed suspensions of *Streptococcus faecalis* Sargent and *Escherichia coli* growing in symbiosis produces putrescine from arginine by way of ornithine. The subsequent fate of putrescine is the subject of a later paper in which further experiments will be discussed.

Hexamine and formamol in acid solution both give rise to formaldehyde and ammonium salts, formamol in addition yielding citrate, and hexamine, on reduction, methylamine. These compounds are potential sources of probable intermediates in the synthesis of alkaloids of the tropane group and their effect on injection bears out this conclusion. None of the remaining substances injected gives a significant increase of alkaloid content. Urea appears to inhibit the production of alkaloid, and this observation was also previously made in experiments on urea feeding [Cromwell, 1937]. It is considered that accumulation of urea in the tissues may influence adversely the formation of ornithine from arginine, thus inhibiting the production of putrescine.

Methylamine, dimethylamine, trimethylamine and choline are each responsible for stimulation of alkaloid production when injected with glucose. Ackermann & Schütze [1910, 1911] found methylamine and trimethylamine as the result of bacterial action on choline, and if methylamine is an intermediate in hyoscyamine synthesis it may be derived from choline, which is known to be present in the root of *Atropa belladonna* [Kunz, 1885]. Glycine, however, may provide methylamine on decarboxylation, and this amino-acid also causes a slight increase of alkaloid on injection. Lysine and proline both show a stimulating effect. Ammonium sulphate and asparagine give a positive result which may be due to their indirect effect on amino-acid production. Cadaverine, betaine, succinimide and potassium nitrate are virtually without effect.

SUMMARY

1. The seasonal distribution of hyoscyamine in the leaves, stems and roots of *Atropa belladonna* has been investigated. The maximum concentration in leaves, stems and roots occurs in July, the minimum in leaves during the early part of May and in roots in March. Little downward movement takes place from the leaves in autumn.

2. Translocation experiments lead to the view that the root system is the principal seat of alkaloid synthesis. The alkaloid moves as such from the root system to stems, leaves, fruits and seeds.

3. An approximate estimate of the seasonal distribution of carbohydrates has been made in plants growing under normal conditions and in plants deprived of light.

4. The effects of injection of various amines and amino-acids have been studied. Putrescine, arginine, hexamine and formamol stimulate alkaloid synthesis considerably. Putrescine is regarded as an intermediate in the synthesis of hyoscyamine.

5. Plants sprouting in darkness give a high yield of alkaloid and volatile bases in both roots and etiolated shoots.

6. The significance of these results is discussed.

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The Role of Putrescine in the Synthesis of Hyoscyamine

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When putrescine (tetramethylenediamine) is introduced directly by injection into plants of *Atropa belladonna* and *Datura stramonium*, a considerable increase in the content of hyoscyamine has been found to occur. This result has been interpreted as an indication that putrescine is an intermediate in the synthesis of hyoscyamine [Cromwell, 1943]. If this interpretation is correct it becomes of interest to attempt to discover the manner in which putrescine provides its contribution to the alkaloid molecule. If one bears in mind the use of succindialdehyde in the synthesis of tropinone by Robinson [1917] and Schöpf & Lehmann [1935], the most probable conclusion would be that putrescine first gives rise to succindialdehyde as the result of oxidation. Alternatively, putrescine may undergo partial oxidation with the formation of an aminoaldehyde (γ-aminobutyraldehyde).

The occurrence of a diamine-oxidase system in animal tissues has been reported by Best & McHenry [1930], Zeller [1938, 1940], Zeller, Birkhauser, Mislin & Wenk [1939], and in bacteria by Gale [1942]. Zeller [1938] showed that this enzyme brings about deamination of histamine, putrescine, cadaverine and agmatine, and Gale [1942], working with washed suspensions of *Ps. pyocyanea*, found that at pH 7·5 the oxidation of putrescine, cadaverine and agmatine proceeds to completion. Zeller [1940] reported inhibition of the diamine-oxidase system by cyanide, semicarbazide, choline, monoamines, and over-optimal concentrations of substrate. It was thought that an enzyme bringing about oxidation of putrescine might exist in the tissues of *Atropa*, and accordingly an attempt was made to prepare an active extract from this plant.

EXPERIMENTAL

(1) *Preparation of crude extract from* Atropa *roots, leaves and etiolated shoots.* Freshly gathered material (500 g.) was washed and finely minced with silver sand in a mincing machine. The pulp was transferred to a muslin bag and pressed out in a strong hand-press. Sufficient glycerol was added to the press-juice to make 5% and the press-cake treated with the glycerol solution for ½ hr. The extract was again filtered through muslin and the residue pressed out. The combined press-juice and filtrate was centrifuged

for 15 min. at 2000 r.p.m., the supernatant solution poured off, dialyzed for 12 hr. at 2° and brought to the required pH (7·2 or 6·8).

(2) *Determination of the deaminating activity of extracts.* A preliminary examination of the deaminating activity was made by use of the colorimetric method described by Zeller [1940], who employed potassium indigo disulphonate as an indicator in the diamine-oxidase system, and found that the amount of dye decolorized was proportional to the amount of diamine oxidized.

25 ml. of the enzyme extract of roots were placed in each of five 200 ml. conical flasks (*a*, *b*, *c*, *d*, *e*), and 10 ml. M/15 phosphate buffer (pH 7·2), 2 ml. toluene and 1 ml. 0·05 % solution of the dye were all added. Flasks *a*, *b*, *c* each received 5 mg. putrescine dihydrochloride. Flask *c* was boiled for 30 sec. before addition of reagents; *d* and *e* served as controls.

All flasks were closed with rubber stoppers, placed in the incubator at 25°, and shaken periodically. After 5 hr. incubation, decolorization was almost complete in flasks *a* and *b*, but had also progressed in the controls to some extent. No decolorization took place in *c*. This experiment gave some indication of the activity of a diamine-oxidase system in the extract. As a manometric determination of enzyme activity was impracticable, ammonia was determined after incubation for 24 hr. at 25° by aeration followed by titration and Nesslerization.

25 ml. of the extract (pH 7·2 or 6·8) were pipetted into each of seven 200 ml. conical flasks *a*, *b*, *c*, *d*, *e*, *f* and *g*. 10 ml. M/15 phosphate buffer of the required pH were added, followed by 2 ml. toluene. Flasks *a*, *b* and *c* each received 2 ml. of a solution of putrescine dihydrochloride, the equivalent of 5 mg. Flask *d*, which had been boiled for 30 sec., received 5 mg. putrescine dihydrochloride. Flasks *e* and *f* were used as controls and *g* received 5 mg. putrescine dihydrochloride and 2 ml. M/50 KCN. Oxygen was introduced into the flasks in alternate experiments, the flasks closed with rubber stoppers and incubated at 25° for 24 hr. After incubation, *c* was examined for the presence of bacteria, and prior to aeration 5 mg. putrescine dihydrochloride were introduced into the controls. 5 ml. saturated

Table 1. *Deaminating activity of crude extracts of* Atropa

		Ammonia (mg.) and aldehyde (+ etc.) present after incubation of flask					
		a	*b*	*d*	*e*	*f*	*g*
		(*a*) Root extract					
(1) Oxygen, pH 7·2	(Ammonia)	3·02	3·01	2·02	1·97	1·99	1·93
	(Aldehyde)	+ +	+ +	+	+	+	+
(2) Air, pH 7·2		2·62	2·79	2·17	1·95	1·96	2·07
		+ +	+ +	+	+	+	+
(3) Oxygen, pH 7·2		3·09	3·11	2·19	2·07	2·10	2·05
		+ +	+ +	+	+	+	+
(4) Air, pH 7·2		2·24	2·25	2·04	1·85	1·87	1·82
		+ +	+ +	+	+	+	+
(5) Oxygen, pH 6·8		2·24	2·36	2·10	1·98	1·97	1·98
		+	+ +	+	+	+	+
(6) Air, pH 6·8		2·27	2·23	2·22	2·09	2·05	2·04
		+ +	+	+	+	+	+
		(*b*) Extract from etiolated shoots					
(1) Air, pH 7·2		3·38	3·30	3·14	3·13	3·09	3·09
		+ +	+	+	+	+	+
(2) Oxygen, pH 7·2		3·26	3·21	3·04	2·97	2·96	2·99
		+ +	+ +	+	+	+	+
(3) Air, pH 7·2		3·11	3·13	3·04	3·04	2·99	2·97
		+	+	+	+	+	+
(4) Oxygen, pH 7·2		3·14	3·11	2·91	2·86	2·88	2·89
		+ +	+	+	+	+	+
		(*c*) Extract from leaves					
(1) Oxygen, pH 7·2		2·18	2·14	2·16	2·09	2·13	2·09
		+ +	+	+	+	+	+
(2) Air, pH 7·2		2·02	1·92	2·02	1·97	1·93	1·95
		+	+	+	+	+	+
(3) Oxygen, pH 7·2		2·30	2·26	2·31	2·22	2·21	2·21
		+	+	+	+	+	+
(4) Air, pH 7·2		2·19	2·22	2·24	2·17	2·19	2·14
		+	+	+	+	+	+

K_2CO_3 solution were run into each flask and aeration carried out for 40 min. at the temperature of the boiling water-bath. Approximately equal volumes of air were drawn through each of the flasks by means of aspirators, and the ammonia aerated into 25 ml. N/100 acid and back-titrated with N/100 alkali from a micro-burette. After titration the value was checked by the use of Nessler's solution.

Aldehyde production in a series of parallel experiments was gauged by the use of 2 : 4-dinitrophenylhydrazine in 2N HCl. After removal of protein by 10% $CCl_3.COOH$, 5 ml. of a saturated solution of 2 : 4-dinitrophenylhydrazine in 2N HCl were added and the tubes allowed to stand for ½ hr. After this time the tubes were shaken and 10 ml. of the solution transferred to a comparator tube. 5 ml. 20% NaOH were now added and the depth of colour compared in control and experimental tubes.

From Table 1 it will be observed that the presence of introduced putrescine results in an increased formation of ammonia and aldehyde in extracts of root and etiolated shoots but not to any extent in leaf extracts. It thus appears that putrescine is deaminated in the tissues of roots and etiolated shoots, with the formation of ammonia and an aldehyde. The activity of the extract is greater at pH 7·2 than at pH 6·8 and greater in oxygen than in air.

Deamination is inhibited by the presence of cyanide and proceeds independently of bacterial activity.

(3). *Examination of leaves and upper stems of* Atropa belladonna *and* Datura stramonium *for putrescine.* If putrescine is an intermediate in the synthesis of hyoscyamine it would be expected that it might be detectable in the tissues in certain circumstances. The probable occurrence of putrescine in tissues of *Datura stramonium* has been reported by Ciamician & Ravenna [1911], and the presence of a diamine in the leaves of *Atropa belladonna* is mentioned by Greenish [1933]. The tetramethyl derivative of putrescine was isolated in 1907 by E. Merck from *Hyoscyamus muticus* [Henry, 1924].

An examination of leaves and upper stems was therefore made, by the method given below, with the object of isolating the diamine.

2 kg. of tissue were finely minced, treated with 1 l. of saturated ether-chloroform water (4 vol. ether : 1 vol. chloroform) and allowed to stand for 2 hr. The pulp was transferred to a muslin bag, pressed out, and the press-cake extracted twice with water (250 ml.). The combined extracts were heated to 90° and the coagulable protein and tissue debris removed by centrifuging. The clear solution was concentrated to 300 ml. under reduced pressure and treated with lead acetate. The precipitate was filtered off and the lead removed from the filtrate with H_2S. The filtrate was further concentrated, made slightly alkaline with NaOH and the alkaloids shaken out with 3 portions of 100 ml. ether-chloroform mixture. After removal of alkaloids, the alkaline solution was gently warmed under reduced pressure to remove dissolved ether and ammonia. Histidine and arginine were precipitated (at pH 7·2 and 12 respectively) with $AgNO_3$, and the lysine fraction with phosphotungstic acid. The lysine precipitate was suspended in water and the bases regenerated with $Ba(OH)_2$. The filtrate was made slightly acid with dilute HCl and evaporated to dryness *in vacuo*. The residue was next made slightly alkaline with Na_2CO_3, extracted with absolute ethanol, filtered, and the bases precipitated by addition of a saturated solution of $HgCl_2$ in ethanol. After filtration, the precipitate was suspended in water, and mercury removed by H_2S. To the filtrate a few drops of dilute HCl were added, the solution concentrated to 2 ml. and excess of absolute ethanol added. The crystals which separated on standing were removed by filtration, dissolved in the minimum amount of water, reprecipitated with absolute ethanol, and finally recrystallized from absolute methanol. Crystals were obtained which melted at 289°. (Putrescine dihydrochloride has m.p. 290°.) (Found: N, 17·1%. Calc. for putrescine dihydrochloride, N, 17·4%.) The yield of putrescine dihydrochloride from leaves and upper stems of *Atropa belladonna* gathered in May and June was nil, from leaves gathered in August 0·01 g., and from *Datura stramonium* gathered in August 0·022 g. On benzoylation and recrystallization from ether, 10 mg. of the dihydrochloride yielded 15 mg. of crystals which melted at 175–176°. (Found: N, 4·82%. Calc. for dibenzoyl putrescine, N, 4·73%.) A mixed m.p. with authentic dibenzoyl putrescine (m.p. 177°) showed no depression.

On the assumption that putrescine was not lost in manipulation, it is concluded from this examination that at certain periods the diamine shows slight accumulation in leaves and upper stems. During the early part of the growing season accumulation may be prevented by the activity of diamine-oxidase.

Roots, leaves and etiolated shoots of *Atropa* were also examined for the presence of methylamine after ammonia had been removed by yellow HgO, but it was not possible to detect this amine with any degree of certainty. Formaldehyde also was absent from these tissues (dimedon test). Plants which had been injected with choline, betaine, hexamine, trimethylamine, dimethylamine and glycine likewise failed to show the presence of methylamine. Ammonia and aldehyde production, however, was intensified in plants injected with glycine, and also in cut shoots which were allowed to take up this amino-acid from dilute solution.

DISCUSSION

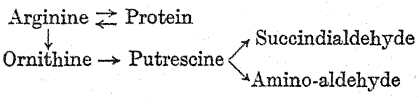
From the results of earlier experiments on *Atropa belladonna* it has been considered that putrescine forms a link in the chain of reactions leading to the synthesis of hyoscyamine and that the most active synthesis takes place in the root system.

The occurrence of an enzyme system which brings about the oxidative deamination of putrescine (and

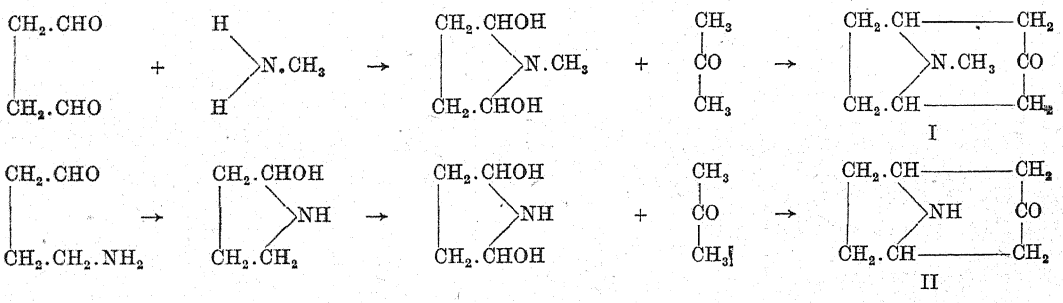
probably cadaverine) in roots and etiolated shoots adds further weight to the above considerations, and gives some degree of biological support to laboratory syntheses of certain members of the tropane group of alkaloids. The optimum pH for diamine-oxidase activity (7·2), however, is higher than that of the cell sap (5·8–6·2), but it does not follow that the pH of the protoplasm of certain cells or tissues must necessarily be the same as that of the cell sap. Fluctuations in pH values may induce the activity of enzymes which are adaptive and thus accumulation of acidic or basic groups may be prevented. For example, Gale [1940] has shown that the amino-acid-decarboxylase activities of bacteria are in some cases strictly adaptive.

Arginine, the precursor of putrescine, either arises as an intermediate in the synthesis of protein or is produced from protein breakdown. In either case it is considered that arginine may be diverted to the production of putrescine.

Partial oxidation of putrescine with the elimination of one molecule of NH_3 would give rise to an amino-aldehyde, and succindialdehyde would be formed as the result of oxidation of both amino groups. Although neither of these aldehydes was isolated, it is presumed that one or both are present when putrescine is subjected to oxidation, in view of the positive reaction with 2 : 4-dinitrophenyl-hydrazine. The above reactions are represented in the following scheme:

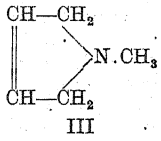


In accordance with the findings and suggestions of Robinson [1917, 1936], succindialdehyde and the amino-aldehyde might contribute to the molecules of tropinone (I) and nor-tropinone (II) according to the scheme outlined below:

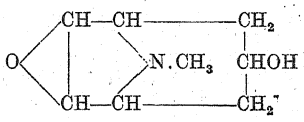


Tropinone and nor-tropinone on reduction would yield tropine and nor-tropine, and linkage with tropic acid would then give hyoscyamine and nor-hyoscyamine. If an active acetone derivative (from carbohydrate via citric acid) were not immediately available, it is suggested that the volatile bases N-methyl-pyrrolidine or N-methyl-pyrroline (III) would be produced. Similarly if cadaverine is present (from lysine) and undergoes oxidative deamination to produce glutardialdehyde or an amino-aldehyde, a pyridine ring would result.

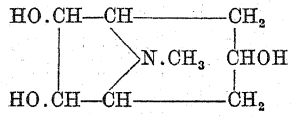
The two alkaloids hyoscine and meteloidine, both closely related to hyoscyamine, occur together in *Datura meteloides* and on hydrolysis yield the bases scopine (IV) and teloidine (V) respectively. The occurrence of N-methyl-pyrroline in the leaves of *Atropa belladonna* and *Datura* spp. suggests a possible relationship between this base and scopine and teloidine. If, in N-methyl-pyrroline, the double bond is represented as occurring between carbon atoms 3 and 4 (III), linkage with oxygen would give part of the structure of scopine and subsequent addition of a molecule of water would then yield part of the teloidine molecule. The origin of the double bond might possibly be associated with a dehydrogenase similar to if not identical with succinic dehydrogenase.



III



IV



V

As no evidence for the presence of methylamine in the tissues of *Atropa belladonna* and *Datura stramonium* has yet been obtained, the origin of the methyl group attached to the N atom is still obscure. The presence of glyoxylic acid, however, is suspected, and it might possibly be responsible for the methylation of the N atom in the tropane group of alkaloids. Alternatively, an entire methyl group may be transferred from a highly methylated

compound such as betaine or choline [Challenger & Higginbottom, 1935; Challenger, 1942]. The formation of tetramethyl putrescine in *Hyoscyamus muticus* could be explained by assuming either that dimethylamine reacts with succindialdehyde, or that glyoxylic acid (or formaldehyde) brings about direct methylation of putrescine.

SUMMARY

1. An enzyme capable of oxidizing putrescine with the formation of ammonia and an aldehyde has been shown to exist in the tissues of *Atropa belladonna*.

2. The enzyme is located chiefly in the root system, but it also occurs in etiolated shoots.

3. Putrescine has been isolated in small amounts from leaves and upper stems of *Atropa belladonna* and *Datura stramonium*.

4. The possible role of putrescine in the synthesis of hyoscyamine and related alkaloids is discussed.

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Studies in the Biochemistry of Micro-organisms

72. *GENTISYL ALCOHOL* (2:5-*DIHYDROXYBENZYL ALCOHOL*), A METABOLIC PRODUCT OF *PENICILLIUM PATULUM* BAINIER

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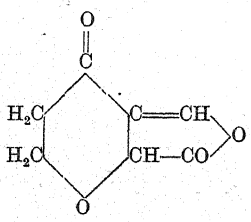
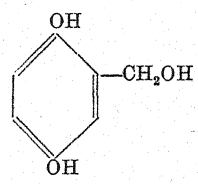
Workers in this laboratory [Birkinshaw, Bracken, Michael & Raistrick, 1943] have recently described the isolation of patulin, $C_7H_6O_4$, believed to be anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid (I), from cultures of *Penicillium patulum* Bainier grown on Raulin-Thom medium. Patulin is a strong anti-bacterial substance. It completely inhibits the growth of a large series of Gram-positive and Gram-negative bacteria at concentrations of 1 : 33,000 to 1 : 100,000. It is also a powerful anti-fungal agent, since it completely inhibits, at a concentration of 1 : 400,000, the growth of a number of species of *Pythium* which are plant pathogens [Anslow, Raistrick & Smith, 1943].

Birkinshaw *et al.* [1943] recorded that the ethereal mother liquors, from which patulin had been isolated, still contained other metabolic products of *P. patulum*. One of these products, which forms the subject of the present communication, has now been isolated, in yields of 0·6 g./l. of culture filtrate, and identified as *gentisyl alcohol* (2 : 5-*dihydroxybenzyl alcohol*) (II). Gentisyl alcohol has only slight anti-bacterial activity against *Staphylococcus aureus*.



I



II

It is a curious fact that, so far as we can ascertain, a compound of this simple chemical constitution has not been described previously, although the corresponding aldehyde, gentisic aldehyde, and the corresponding acid, gentisic acid, which is also formed by *P. patulum*, have been known for a long time.

The chemical constitution of the new metabolic product was established as follows. It has the empirical formula, $C_7H_8O_3$. On long continued reduction with Pd-norite-H_2 it is converted into toluquinol. Oxidation of the dimethyl ether of the metabolic product with $KMnO_4$ gives 2 : 5-dimethoxybenzoic acid which on demethylation affords gentisic acid (2 : 5-dihydroxybenzoic acid). Finally, gentisyl alcohol was synthesized by the partial reduction of gentisic aldehyde, the initial quick reduction being stopped after the absorption of 1 mol. of hydrogen. Synthetic gentisyl alcohol and the new mould metabolic product have the same m.p. 100°, and a mixture of the two substances melts at the same temperature.

EXPERIMENTAL

Isolation of the new metabolic product. Cultures of *Penicillium patulum* Bainier, strain Ad 77, on Raulin-Thom solution, were prepared in the usual way. After 14 days' incubation at 24° the bulked culture filtrate from 52 flasks had the following characteristics: total vol. 16·14 l.; residual glucose by polarimeter, 0·74%; pH, 5·0; anti-bacterial activity against *Staphylococcus aureus*, complete inhibition at a dilution of 1 : 160, partial inhibition at 1 : 320. The bulked filtrate was evaporated *in vacuo* to 800 ml. and extracted three times with 800 ml. each time of ethyl acetate. The extract was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residual oil (58 g.) which was beginning to crystallize was dissolved in ether and passed through a column of alumina as described by Birkinshaw *et al.* [1943]. Two crops of patulin, wt. 19·42 and 0·88 g., were isolated.

The ethereal mother liquors remaining after separation of the patulin were freed from solvent and the residual oil was dissolved in ether (150 ml.) and hot chloroform (1 l.) was added. The crude metabolic product separated in clusters of needles containing a little oil. The product was collected after 4 days and washed with a little ether. Wt. 6·98 g., m.p. 98–99·5°. The mother liquor, after standing in an open flask for a considerable time, deposited a second crop of needles mixed with some oil. Wt. 2·90 g., m.p. 84–96°.

Purification of the crude metabolic product presented some difficulty owing to the tendency of the substance to separate as an oil. It also appears to be somewhat sensitive to heat. No completely satisfactory solvent was found and the method finally adopted was as follows: A portion (4 g.) of the first crop was finely powdered and added to 1 l. of boiling chloroform. The mixture was refluxed for 5 min. and filtered. On cooling, 1·10 g. of almost colourless needles were obtained; m.p. 100° after sintering at 98°. The undissolved portion, 1·97 g., was reworked in subsequent crystallizations.

General properties of the metabolic product. Colourless needles, m.p. 100°, which sublime in a high vacuum at a bath temperature of 70–80°, though not as readily as might be expected of a substituted quinol. (Found: C, 59·87, 60·01; H, 5·89, 6·06; N, nil; OCH_3, nil. $C_7H_8O_3$ requires C, 60·01; H, 5·75%. 2·53 active H atoms in pyridine, 0·41 atoms in anisole. Kuhn-Roth method gives 0·112 mol. acetic acid.)

It is readily soluble in water, ethanol and ether, slightly soluble in benzene and chloroform and insoluble in light petroleum. An aqueous solution has the following properties: neutral to litmus; instantly decolorizes $KMnO_4$; reduces $AgNO_3$ in the cold with mirror formation; gives an evanescent blue colour with aqueous $FeCl_3$, changing almost immediately to yellow; reacts with aqueous iodine with absorption of two atoms of iodine which is slowly lost on titration with thiosulphate; does not react with hydroxylamine even after 24 hr. With 2N NaOH it gives an immediate yellow colour rapidly darkening to strong reddish brown. When heated with 2N H_2SO_4, in which it is readily soluble in the cold, it gives in a few seconds a white amorphous precipitate.

It gives nearly complete inhibition of the growth of *Staphylococcus aureus* at a concentration of 1 : 500, partial inhibition at 1 : 1000 and 1 : 2000 and no inhibition at 1 : 4000.

Catalytic reduction of the metabolic product. Formation of toluquinol. A solution of the metabolic product (0·5 g.) in ethanol (10 ml.) was hydrogenated at ordinary pressure with a palladium-norite catalyst prepared from 0·2 g. of $PdCl_2$ and 0·5 g. of norite. Hydrogenation, which was slow throughout, was discontinued after 8¾ hr. (Uptake of hydrogen, 57·8 ml. (corr.); theor. for uptake of 2H per mol., 80 ml.) After filtering from catalyst the ethanol was removed *in vacuo*. A mass (0·48 g.) of crystals containing some oil remained. This material sublimed readily in a water-pump vacuum at a bath temperature of 110–115°. The sublimate formed large colourless blades, m.p. 127°, setting on cooling and remelting at 127°. Resublimed in a high vacuum the sublimate melted at 128°, alone or in admixture with authentic, sublimed toluquinol, m.p. 128°. (Found: C, 67·39; H, 6·40. Calc. for $C_7H_8O_2$: C, 67·75; H, 6·50%.)

Methylation of the metabolic product. A solution of the metabolic product (2·0 g.) in dry ether (25 ml.) was treated with an excess of ethereal diazomethane for 3 days. On removal of the solvent a reddish oil (2·35 g.) remained, which, on distillation *in vacuo*, gave 1·46 g. of a fairly mobile yellow oil, b.p. 140°/3·3 mm. (Found: OCH_3, 34·1. $C_9H_{12}O_3$ requires, for $2OCH_3$, 36·9%.)

Oxidation of crude dimethoxy derivative. Formation of 2 : 5-*dimethoxybenzoic acid and demethylation*

to gentisic acid. The above oil (1 g.) was suspended in hot water (50 ml.) containing N KOH (1 ml.) and to it was added a solution of $KMnO_4$ (1·5 g.) in hot water (150 ml.). After ½ hr. heating on the water-bath no $KMnO_4$ remained. The solution was filtered, the filtrate evaporated to 25 ml. and acidified to Congo red with 2N HCl (3 ml.). An emulsion was formed which rapidly crystallized, forming colourless needles, wt. 0·55 g., m.p. 72–76°. After recrystallization from water the oxidation product melted at 75–76°, not depressed on admixture with authentic, synthetic 2 : 5-dimethoxybenzoic acid. (Found: C, 59·30; H, 5·60. Calc. for $C_9H_{10}O_4$: C, 59·33; H, 5·53%.)

The acid (0·13 g.) was heated with HI (sp. gr. 1·7; 3 ml.) in a Zeisel apparatus in a stream of CO_2, the bath temperature being maintained at 130–140° during 1 hr. On cooling, fine brownish needles separated. These were collected and washed with conc. HCl. Wt. 0·07 g., m.p. 199–201°. The crystals gave a colourless crystalline sublimate in a high vacuum, m.p. 204° not depressed on admixture with authentic gentisic acid, m.p. 203°. (Found: C, 54·51; H, 4·09. Calc. for $C_7H_6O_4$: C, 54·55; H, 3·92%.)

Synthesis of gentisyl alcohol (2 : 5-*dihydroxybenzyl alcohol*). *Identity with metabolic product.* Gentisic aldehyde (2 : 5-dihydroxybenzaldehyde) (0·5 g.), prepared from salicylaldehyde by the method of Neubauer & Flatow [1907] and dissolved in ethanol (10 ml.), was hydrogenated at ordinary pressure with a palladium-norite catalyst prepared from 0·2 g. of $PdCl_2$ and 0·5 g. of norite. The uptake of hydrogen was fairly rapid. Hydrogenation was discontinued after 50 min. (Uptake of hydrogen, 82·5 ml. (corr.); theor. for uptake of 1 mol. H_2, 81·2 ml.) After filtering from catalyst the ethanol was removed *in vacuo*. A yellow oil (0·57 g.) remained and began to crystallize overnight. The product was recrystallized from chloroform in the same way as is described above for the purification of the crude metabolic product. After two recrystallizations it was obtained as almost colourless needles, m.p. 99° after softening at 95°. It was finally sublimed in a high vacuum. Colourless crystalline sublimate, m.p. 100°, alone or in admixture with the metabolic product. It also gave the same reactions. (Found: C, 60·09; H, 5·79. Calc. for $C_7H_8O_3$: C, 60·01; H, 5·75%.)

SUMMARY

Gentisyl alcohol, 2 : 5-*dihydroxybenzyl alcohol*, occurs together with patulin as a metabolic product of *Penicillium patulum* Bainier. Its isolation, identification and synthesis are described.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **225th Meeting** *of the Biochemical Society was held at King's College, Strand, London, W.C. 2, on Saturday, December 19th, 1942, at 2 p.m., when the following papers were read:*

The Fluorescence of the Tissues in Avitaminosis E.

By T. Moore and Y. L. Wang

It has been previously reported that the uteri of rats restricted to a diet deficient in vitamin E show a marked brown discoloration.* Exposure to the irradiation of a mercury arc lamp, screened with Wood's glass, has now been found to evoke a characteristic yellow or bronze-coloured fluorescence. This fluorescence allows a much more delicate test for vitamin-E deficiency than the inspection of the affected tissues in visible light. Thus in early stages of deficiency strong yellow fluorescence is exhibited before the appearance of noticeable discoloration. Moreover, in the later stages of deficiency fluorescence is displayed throughout the skeletal musculature and in many other tissues of the body, which do not appear grossly abnormal in visible light. No yellow fluorescence was shown by the urinary bladder, brain, lungs or foetuses contained in a discoloured uterus. The yellow fluorescent substance has been concentrated by a process involving hydrolysis of the tissues with barium hydroxide, acidification, extraction with *iso*-butanol, transfer to dilute alkali, precipitation with acid and further purification by mild oxidation. The final product is a brownish yellow amorphous powder, which is soluble in butanol but almost insoluble in ether, chloroform or benzene. It has acidic properties, and in its fluorescence and solubility resembles the 'melanins' formed by the acid hydrolysis of proteins. A similar substance has been isolated from the brown atrophied heart of an aged woman. Further investigations of the properties of the fluorescent substance are in progress.

* Martin, A. J. P. & Moore, T. [1936]. *Chem. Ind.* **55**, 236; [1938] *ibid.* **57**, 973; [1939] *J. Hyg., Camb.*, **39**, 643.

Oxidizing Enzymes and Vitamin C in Tomatoes.

By F. Wokes and J. G. Organ

The concentration of oxidizing enzymes is very high in the skin, fairly high in the flesh and very low in the juice of unripe and ripe tomatoes. The skin remains alkaline (*p*H 8·0–9·6) throughout ripening, but the *p*H of the flesh gradually falls to below 5 as ripening proceeds. The juice appears always to be acid (*p*H 4–5) in both unripe and ripe tomatoes. Destruction of vitamin C by ascorbic acid oxidase liberated from the tissues by grinding them with quartz sand is most rapid in the unripe skin (where 60–80 % may be lost in 1 min.), and rather less rapid in the flesh and juice of unripe green tomatoes. In ripe tomatoes the rate of destruction in the skin is almost as rapid as in unripe tomatoes, but much slower in the flesh and juice. In harmony with these findings, mincing of whole unripe tomatoes causes much more rapid loss of the vitamin than mincing of ripe tomatoes.* The concentration of vitamin C is about twice as high in the skin as in the flesh and juice. The concentration of the vitamin in smaller tomatoes, in which the skin forms a larger proportion of the total weight, may be a little higher, but the difference is not sufficient to discredit the findings of Sansome & Zilva† on polyploidy, as McHenry & Graham‡ had suggested.

* Wokes, F. & Organ, Joan G. [1942]. *Nature*, **150**, 523.
† Sansome, F. W. & Zilva, S. S. [1936]. *Biochem. J.* **30**, 54.
‡ McHenry, E. W. & Graham, M. [1935]. *Biochem. J.* **29**, 2013.

Vitamin B_1 and Toxaemia of Pregnancy. By R. KAPELLER-ADLER and J. A. CARTWRIGHT

Nineteen cases of pre-eclamptic toxaemia, eleven mild and eight severe ones, were treated with vitamin B_1 ('Benerva'). The treatment consisted usually of a course of ten intramuscular injections, 25 mg. of vitamin B_1 having been used on the first day and 10 mg. of vitamin B_1 on each of the following 9 days, but in some cases 10 mg. of vitamin B_1 were given orally instead of injections and a few patients received both.

In no case was there any beneficial effect which could be attributed to the vitamin B_1 and in some severe cases the vitamin seemed to accentuate the toxaemic symptoms.

The vitamin appeared to affect the histamine metabolism in these cases, diminishing the urinary excretion of histamine.

The Bearing of the Dissociation Constant of Urea on its Structure. By J. BELL, W. GILLESPIE and D. B. TAYLOR

The Chemical Findings in a Case of Acute Idiopathic Porphyrinuria. By J. E. KENCH

A case of this unusual condition was admitted to the Manchester Royal Infirmary under the care of Dr F. R. Ferguson, to whom I am indebted for the opportunity of making the investigations now reported.

The patient was a man of 27 whose symptoms were typical of porphyrinuria of the acute idiopathic type. For several years he had suffered from attacks of acute abdominal pain, accompanied by constipation, paralysis of the arms and bilateral wrist drop. Seventeen days after admission to hospital he had another periodic attack which proved terminal.

The urine was dark reddish brown, acid in reaction, and exhibited the two well-defined absorption bands at 580 and 542 mμ characteristic of the metaluroporphyrin complex excreted in this disease.* Acidification with hydrochloric acid caused the absorption bands to move to 600 and 557 mμ, whilst removal of the metal by treatment with sodium amalgam, followed by reoxidation, gave the free porphyrin spectrum. No protoporphyrin could be detected in the urine. The coproporphyrin values were 163 μg./litre before the attack and 128, 43 and 148 μg./litre during the attack, consisting of approximately equal amounts of isomers I and III.

After removal of ether-soluble porphyrins, uroporphyrin was separated by adsorption on Brockmann's alumina at *p*H 6·5, after boiling the urine for 5 min. at *p*H 3·7. The pigment was eluted with dilute ammonia, reprecipitated at *p*H 3·7, esterified in acid alcohol, and purified in the usual way. 191 mg. of uroporphyrin III methyl ester, m.p. 250°, mixed m.p. 249–257°, were obtained, representing 48 mg./litre of urine.

No uroporphyrin I was found, and these findings were confirmed by decarboxylation to the corresponding coproporphyrin III.

A specimen of stools collected in the remission period contained 0·13 mg. coproporphyrin per 100 g. fresh material (predominantly series III). The pigment excretion during the attack had risen to 1·81 mg./100 g. coproporphyrin. No uroporphyrin could be detected at any time.

The liver at autopsy exhibited diffuse passive congestion, the pigment content being 11·2 mg. coproporphyrin III and 50 μg. protoporphyrin (determined by ultraviolet fluorimeter).

No porphyrin could be detected in the blood either spectroscopically or by ultraviolet fluorescence, which may be considered evidence of the rapid and easy excretion of these pigments.†

* Rimington, C. [1938]. *Chem. & Ind.* **16**, 1110.

† Turner, W. J. [1938]. *Arch. Int. Med.* **61**, 762.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **226th Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, W. 12, on Saturday, 20 February 1943, at 12 noon, when the following papers were read:*

Vitamin B_1 in Malt Extract.

By F. Wokes and J. G. Organ

Vitamin B_1 in five different samples of malt extract was estimated by the thiochrome method using the technique of Nicholls, Booth, Kent-Jones, Amos & Ward,* and apparatus calibrated as previously described.† The results of over 200 consecutive assays, summarized in the accompanying table, when compared with microbiological results by Dr E. R. Dawson using the method of Schultz, Atkin & Frey,‡ led to the following conclusions:

(1) Quenching common in vitamin B_1 assays by the thiochrome method causes results to be too low. With malt extract the thiochrome results, if not corrected for quenching, may be less than a tenth of the microbiological values.

Series	Sample	No. of assays	Added aneurin as % of total aneurin	% of sample in solution used	Thiochrome results as per cent. of microbiological results calculating on % recovery of: Added aneurin — Mean	Added aneurin — S.D. of mean	Total aneurin — Mean	Total aneurin — S.D. of mean	Coefficient of variation for single assay based on % recovery of: Added aneurin	Total aneurin
					Unwashed					
1	A	16	21–35	5–40	61·9	± 9·9	82·3	±4·2	63·5	19·7
2	B	16	15–26	5–40	80·5	±13·9	87·3	±4·1	67·2	18·2
3	C	16	16–27	5–40	42·5	± 6·9	75·0	±5·4	64·4	28·0
				Mean	61·6		81·5		65·0	21·9
					Washed with isobutanol to remove quenching substances					
4	A	16	21–35	5–40	68·6	± 7·0	87·5	±1·9	27·9	8·5
5	B	16	15–26	5–40	63·3	±10·1	83·0	±3·0	61·6	14·3
6	B	16	15–26	5–40	100	±16·1	93·0	±1·9	63·0	8·1
7	C	16	16–27	5–40	59·4	± 6·0	92·0	±5·9	39·8	25·1
				Mean	72·8		88·9		48·1	14·0
					Washed and using high concentrations of added aneurin					
8	A	16	52–69	5–40	90·2	± 3·6	93·9	±2·2	14·5	8·7
9	B	16	42–59	5–40	97·0	± 8·8	95·0	±5·0	35·6	20·7
10	B	16	42–59	5–40	75·8	± 3·5	83·4	±1·9	17·9	9·1
11	C	16	42–59	5–40	89·7	± 5·0	94·9	±3·1	22·0	12·7
				Mean	88·2		91·8		22·5	12·8
					Fortified with extra aneurin in form of yeast					
Unwashed										
12	D	13	85–97*	5–20	96·0	± 5·5	99·0	±2·6	17·1	7·9
13	E	6	88–91	5–20	96·8	± 8·5	97·2	±3·5	21·5	8·9
				Weighted mean					18·5	8·2
Washed										
14	E	6	88–91*	5–20	98·0	± 4·9	98·5	±2·0	12·1	5·9

* Includes aneurin added as yeast and as pure aneurin. Standard deviations are for the mean result of all the assays in each series.

(2) Correction for quenching is usually based on the percentage recovery of *added* aneurin. This may deviate widely from the true percentage recovery (i.e. of *total* aneurin

calculated from microbiological results), causing the thiochrome results (*a*) to be much too low, (*b*) to have a very high coefficient of variation.

(3) Part of the quenching can be eliminated by washing the aqueous extract with isobutanol as recommended by Wang & Harris.§ This brings the results nearer to the microbiological values and also reduces the coefficient of variation, but the latter is still about six times the combined coefficients of variation of the thiochrome and microbiological methods as determined by replicate analyses.

(4) Flooding with aneurin (i.e. markedly increasing the proportion of added aneurin) combined with washing with isobutanol reduces the deviation from the microbiological values to a very low amount and the coefficient of variation to about twice the combined coefficients of the two methods.

(5) Since in over 200 consecutive assays the coefficient of variation was always greatly reduced by calculating results on recoveries of *total* instead of *added* aneurin the latter causes much of the experimental error of the thiochrome method. Low results on certain foodstuffs obtained by other workers using this method are probably largely due to inadequate compensation for quenching, which may help to explain deviations between thiochrome and biological results already observed by Harris & Wang.‖

(6) Comparison of aneurin content with diastatic power (old Lintner) gave: D.P. 12, 3·85 μg./g.; D.P. 112–132, 5·6–5·7 μg./g., in agreement with earlier findings of Bacharach & Allchorne¶ that a higher D.P. was associated with higher content of vitamin B complex.

* Nicholls, J. R., Booth, R. G., Kent-Jones, D. W., Amos, A. J. & Ward, H. H. [1942]. *Analyst*, **67**, 15.
† Wokes, F. & Still, B. M. [1941]. *Chem. & Ind.* **60**, 722; Wokes, F., Organ, J. G. & Jacoby, F. C. [1942]. *Proc. Biochem. Soc.* **36**, vii.
‡ Schultz, A., Atkin, L. & Frey, C. N. [1942]. *Industr. Engng Chem.* (Anal. ed.), **14**, 35.
§ Wang, Y. L. & Harris, L. J. [1939]. *Biochem. J.* **33**, 1356.
‖ Harris, L. J. & Wang, Y. L. [1941]. *Biochem. J.* **35**, 1050.
¶ Bacharach, A. L. & Allchorne, E. [1928]. *Biochem. J.* **22**, 313.

The Vitamin P Activities of some British Fruits and Vegetables. By M. E. Coates and A. L. Bacharach

By the method previously described* biological tests have been carried out to compare the vitamin P activities of between 20 and 30 different vegetables or fruits and that of a provisional standard preparation, a water-soluble concentrate of citrus origin. Activities ranging from 0 (lentils) to 132 units (spinach) per 100 g. have been found. Full assays were carried out on 11 different vegetable products, which were contemporaneously tested for ascorbic acid content. There was no correlation. The vitamin P activity also bore little if any relationship to the colour of the product tested. The vitamin P content of peas was doubled after a few days' germination, but the ascorbic acid content increased nearly 20-fold.

* Bacharach, A. L., Coates, M. E. & Middleton, T. R. [1942]. *Biochem. J.* **36**, 407; Bacharach, A. L. & Coates, M. E. [1942]. *Analyst*, **67**, 313.

The State of Tyrosine in Egg Albumin and Insulin as shown by Spectrophotometric Titration. By J. L. Crammer and A. Neuberger

The change of the ultra-violet absorption spectrum of tyrosine with *p*H has been utilized to estimate the *pK* of the phenolic group in tyrosine, in diiodo-tyrosine and in proteins. The *pK* obtained for the two amino-acids agrees well with the values obtained by other methods. In insulin it can be shown that the phenolic groups are free, i.e. able to ionize,

and that the pK is displaced towards higher values. This is due to the negative charge of the molecules in alkaline solution. In negative egg albumin the phenolic groups cannot ionize; this is probably due to the fact that the phenolic groups are bound in a linkage, possibly a hydrogen bond. On denaturation this bond is broken and the phenolic groups ionize normally. It is believed that such linkages of phenolic groups are of major importance for the structures of native protein molecules.

An Examination of the Relationship between the Bacteriostatic Activity and the Oxidation-Reduction Potentials of Substituted Quinones. By J. E. PAGE and F. A. ROBINSON

Several workers have suggested that there might be a simple relationship between the oxidation-reduction potential of a substance and its antibacterial and related activities, and it was considered that the series of highly antibacterial quinones tested against strains of *Staph. aureus* and of *Bact. coli* under standard conditions by A. E. Oxford,* appeared to offer an opportunity of testing this theory.

We† examined the behaviour at the dropping mercury electrode of a number of mould metabolites and related quinones in acetate-buffered 75 % alcoholic solution and calculated the oxidation-reduction potentials (E'_0) at pH 7·0 of these substances from their half-wave potentials. We failed to find a direct relationship connecting the reduction potential of these quinones with their bacteriostatic activity against strains of *Staph. aureus* and *Bact. coli*, but observed that, for *Staph. aureus*, the reduction potentials of all the active quinones examined to date fell between certain limits.

* Oxford, A. E. [1942]. *Chem. & Ind.* **61**, 189.

† Page, J. E. & Robinson, F. A. [1943]. *J. chem. Soc.* (in the Press).

A Rat Growth Method for the Assay of Pantothenic Acid. By J. S. D. BACON and G. N. JENKINS

A supplement to a basal diet and synthetic vitamins was sought which should provide the hitherto unidentified growth factors of the vitamin B complex but should contain no pantothenic acid. Acid and alkaline hydrolysates of liver extracts did not fulfil the above condition, as the pantothenic acid was not destroyed completely. Wheat germ, however, fed at a level of 0·5 g. per day fulfilled these conditions approximately. A satisfactory curve was constructed relating growth of rats to dosage of pantothenic acid over a range of 0–100 μ daily given to rats receiving basal diet, synthetic B vitamins and wheat germ.

This result provides a rat growth method for assaying pantothenic acid in foods.

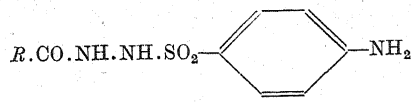
Propylene Glycol as a Solvent for the Intravenous Injection of DOCA. By E. P. SHARPEY-SCHAFER

'March' Haemoglobinuria in a Soldier. By H. S. MITCHELL and R. A. PALMER

Phenolphthalein Phosphate as a Reagent for Studying the Phosphatase Content of Organisms. By J. BRAY and E. J. KING

Concentration of Sulphanilamide Derivatives in Bile of Rabbits. By MARY BARBER, J. H. DIBLE and G. A. D. HASLEWOOD

A compound of cholic acid and sulphanilamide (sulphacholazine) having the formula



(R = cholyl) was prepared by one of us* in the hope that it might be concentrated in the bile. This compound has considerable bacteriostatic activity *in vitro* against *Strep. haemolyticus* and *Strep. pneumoniae*, but little or none against members of the coliform group of organisms.

A series of rabbits have been injected intravenously with sulphacholazine, sulphanilamide and sulphapyridine respectively. Attempts have been made to estimate the concentration of each substance reached in the bile by means of the diazo reaction after first precipitating the bile with alcohol. This reaction produced no colour with bile from normal rabbits, and the red colour which developed in the bile of the experimental animals was compared in a colorimeter with that of a known standard of the compound originally injected. These experiments indicate that sulphacholazine reaches a high concentration in the bile of rabbits, whereas sulphanilamide and sulphapyridine are never present in the bile except in very small amounts. Attempts to demonstrate that bile containing high concentrations of sulphacholazine is more bactericidal *in vitro* than normal rabbit bile have so far failed, owing to the highly strepticidal nature of the latter.

* Haslewood, G. A. D. [1941]. *Biochem. J.* **35**, 1307.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held at the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1, on Saturday, 27 March* 1943, *at 2 p.m., when the following papers were read:*

The Measurement of Fluorescence Intensity. By P. Ellinger and M. Holden

Examination of physical conditions governing measurement of fluorescence intensity carried out to develop a fluorimeter suitable for routine purposes reveals that the photoelectric cells are most satisfactory analysers. Relative arrangement of analyser, container of fluorescent solution and light source should be in straight line so that light intensity measured is dependent exclusively on concentration of fluorescent material. Primary light can be excluded from analyser by suitable filters and should be monochromatic to obtain absorption corresponding to Beer's law. 365mμ mercury line is suitable light source. Interference with measurement caused by fluorescence maximum, mainly dependent on absorption, can be avoided by using narrow containers and high primary light intensity. Measurement can be made either with one photocell by measuring galvanometer deflexion and relating it to primary light intensity or by compensating methods involving two photocells and using the galvanometer as a null instrument, balancing either the light by altering the aperture of primary or secondary beam, or the photocell currents with the potentiometer. Comparative results (table) show potentiometer method superior in range, speed and accuracy. A fluorimeter designed on these principles is described.

Comparative results obtained on quinine sulphate with various fluorimeter arrangements

		Balancing methods		
		Altering the aperture of		
	Direct deflexion measurement	Primary beam	Secondary beam	Potentiometer
Range	1 : 500	1 : 32	1 : 16	1 : 128
Speed (time for 10 readings)	24·5′	14·5′	15′	9′
Standard deviation in % of mean (10 readings)	1·2–5·0	0·17–1·5	0·54–1·4	0·06–2·3

A Chromatographic Separation of Coproporphyrin from Uroporphyrin; an Improved Method for the Preparation of Uroporphyrin from Pathological Urines. By C. Rimington

In urines from cases of acute porphyry, the porphyrins are accompanied by large quantities of dark brown or red pigments which are very difficult to remove. All adsorbents so far recommended are used to adsorb the porphyrins in the region of their isoelectric point, at which reaction the accompanying pigments are also retained. Attempted precipitation of the crude porphyrin by means of acetic acid is not always successful and any precipitate obtained contains a mixture of coproporphyrin and uroporphyrin which is troublesome to separate.

Kieselguhr possesses the remarkable and very desirable property of adsorbing both coproporphyrins and uroporphyrins from solution *in dilute hydrochloric acid* whilst rejecting almost entirely the dark brown, non-porphyrin pigments. Elution by buffer solutions may be made selective by taking advantage of the fact that uroporphyrins I and III are removed at *p*H 5·25, while coproporphyrins I and III, no doubt by virtue of their higher isoelectric point, are not eluted until the *p*H of the buffer reaches 5·5 or 6·0. It has not been possible to separate the individual isomers of each porphyrin from each other by this means.

As a preparative procedure the method was applied to a very dark specimen of urine from a case of acute porphyry as follows: To the urine, hydrochloric acid was added to a concentration of 1 % and the mixture passed through a Tswett column of acid-washed kieselguhr (B.D.H.). The deeply coloured filtrate was free from porphyrin. The column was now washed successively with 1 % HCl, glacial acetic acid (which removed a little more brown pigment and a small quantity of porphyrin) and water. Molar acetate buffer *p*H 5·25 was now used and removed the uroporphyrin as a deep red solution substantially free from other pigment. Upon acidification, the porphyrin precipitated readily and was removed for esterification in the usual way.

A Method for the Quantitative Determination of Uroporphyrin in Pathological Urines. By C. Rimington

Great difficulties attend the quantitative determination of uroporphyrin. Adsorption methods are usually attended by loss and do not remove accompanying dark pigments. Uroporphyrin III is sparingly soluble in ethyl acetate under strictly controlled conditions of *p*H, but uroporphyrin I is not extractable from urine by this or any other known solvent immiscible with water.

A satisfactory quantitative procedure has been elaborated based upon the selective adsorption of the porphyrins from acid solutions (1 % HCl) by kieselguhr.

In order to convert all porphobilinogen or other precursors into porphyrin, hydrochloric acid is added to the urine to a concentration of 1 % and the mixture left overnight or, less desirably, heated for 1 hr. in the water-bath. An aliquot (usually 1–3 ml.) containing 2–20 μg. of porphyrin is diluted in a centrifuge tube with 1 % HCl to about 5 ml., 0·7 g. acid-washed kieselguhr (B.D.H.) added and after vigorous shaking the mixture centrifuged. The adsorbent is washed by shaking successively with two lots of 1 % HCl and one of water. The porphyrin is now eluted by stirring with three successive lots of about 7 ml. each of 0·1 *N* NaOH and the combined eluate made up to 25 ml. Small particles of kieselguhr may render this alkaline solution turbid, but if left overnight they sink, leaving a perfectly clear and almost colourless supernatant, a portion of which is suitably diluted with 0·1 *N* NaOH and the porphyrin content determined in a fluorescence comparator (optimal porphyrin concentration about 0·25 μg./ml.). This measures the combined uroporphyrin and coproporphyrin. Coproporphyrin is determined separately by the ether extraction method, either in the eluate or in the original urine, and the uroporphyrin calculated by difference. As an illustration of the method, the daily output of uroporphyrin and coproporphyrin by a case of acute porphyrinuria is presented. The period of 23 days includes an attack and remission.

The Toxicity of Pure Vitamin A. By T. Moore and Y. L. Wang

It has long been known that greatly excessive doses of vitamin A concentrate are poisonous to rats, but opinions have been divided as to whether the toxicity is due to vitamin A itself or to some accompanying impurity. Tests of the toxicity of crystalline vitamin A

acetate ($E\,\frac{1\%}{1\,cm.}=1500$ for acetate) were made possible through the kindness of British Drug Houses Ltd., in providing a generous sample. Doses of about 25,000–50,000 i.u. daily to young rats of 69–79 g. body weight invariably caused fractures of the bones after periods of 8–20 days. Doses of about 50,000 given to adult pregnant rats did not cause breakages of the bones within the period of the experiment, but gave rise to fatal uterine haemorrhage, as originally described by Hartwell in rats given excess of cod-liver oil. This phenomenon appears to be one manifestation of the tendency to haemorrhage in hypervitaminosis A. Other forms of haemorrhage are subcutaneous or intramuscular haemorrhage which occurs frequently in the legs; massive and rapidly fatal haemorrhage under internal membranes, such as the pericardium; or excessive haemorrhage as the result of minor external injury. In some respects the tendency to haemorrhage in hypervitaminosis A has a strong superficial resemblance to that found in scurvy.

The Metabolism of Azo Compounds. By L. A. Elson and F. L. Warren

Crystallization on Foam. By R. Bader and F. Schütz (introduced by W. V. Thorpe)

When solutions of bile salts are partly converted into foam, the acids crystallize in different fractions of the foam. Crystals of bile acids can readily be obtained from crude commercial specimens of bile salts. Some conditions of crystallization and a number of principles of the foam method are discussed, which have been derived from measurements of foam stability* of the substances involved.

* Schütz [1937]. *Nature, Lond.*, **139**, 629; [1938], *C.R. Soc. Physiol. Suisse*, **46**; [1942], *Trans. Faraday Soc.* **38**, 85, 94.

Correlation of the Oxygen Capacity, Iron Content and Absorption Spectrum of Haemoglobin of Normal Subjects. By H. M. Jope, E. M. Jope and J. R. O'Brien

It has become increasingly evident that the word 'haemoglobin' as applied to whole blood could not be adequately defined. We have accordingly set out to determine the extent of correlation of its chief properties—O_2 and CO combining power, iron content and absorption spectrum—as found in whole blood, and if possible to relate our results to the properties of some definable solid haemoglobin preparation. The results from the first 25 normal human bloods examined are reported here.

	Fe : O_2 c.c. O_2/1 g. Fe	Fe : Extinct. coeff. Soret band $E^{1\,g.\,atom\,Fe/l.}_{1\,cm.} \times 10^{-3}$	O_2 : Extinct. Soret band $E^{1\,mol.\,O_2/l.}_{1\,cm.} \times 10^{-3}$
Theoretical			
1 atom Fe : 1 mol. O_2	401	—	—
Our mean	396	132	138
Extreme range	367–418	127–141	125–152
Standard deviation, as % of mean	3·3%	3·2%	5%

The relation of absorption spectra of carboxy- to oxyhaemoglobin appears to be even more irregular. All the experimental methods used are reproducible to within ±0·2–0·5%. The discrepancies in the exact correlation would therefore seem to be very largely real: it is unlikely that they are due to systematic errors in the methods. Our results indicate that it is not adequate to define the absolute value of one property in terms of another, and hence the standard for, say, a photometric method in terms of a gasometric method.

The possible uncertainty of defining the water content of crystalline preparations of proteins made it desirable to relate these data to a preparation of haemoglobin dried under standard conditions. The procedure of Morrison & Hisey,* with a few modifications, gave dried horse haemoglobin, which redissolved easily in water. This clear solution carried 409 c.c. O_2/g. Fe, which indicates that every iron atom present was capable of carrying oxygen.

* Morrison, D. B. & Hisey, A. [1935]. *J. biol. Chem.* **109**, 23.

Further Observations on the Excretion of Coproporphyrin in Normal Subjects.

By E. M. Jope and J. R. O'Brien

1. *Definition of standard.* From a study of synthetic and natural coproporphyrins I and III the absolute extinction coefficients for the free porphyrins in 0·5 % HCl are defined:

$$548\ \mathrm{m}\mu = E^{1\%}_{1\ \mathrm{cm.}} = 260, \quad 401\ \mathrm{m}\mu = E^{1\%}_{1\ \mathrm{cm.}} = 8100.$$

III is equivalent to I within $\pm 1\%$.

2. *Normal range of coproporphyrin excretion.* (*a*) *Urine*: in 3-day collections, below 100 μg./day, though isolated 24 hr. values may be 100–150 μg./day. The lower limit so far observed is 10 μg./day. (*b*) *Faeces*: 3-day averages are 150–350 μg./day: shorter collection periods give results too erratic to be usable. Any one individual varies less than this overall normal range.

3. The excretion of coproporphyrin in urine throughout the 24 hr. is irregular: estimations on single urine specimens are therefore of little value.

4. Maechling* reports increased coproporphyrin in pathological urines after standing 2 hr. with $\frac{1}{10}$ vol. conc. HCl. We have confirmed this occasionally in pathological cases but have not observed any increase in normals.

5. Evidence in the literature suggests that the normally excreted coproporphyrin is largely the I isomer, but that some III may occur. I is more easily recognized and Fischer originally missed the III in the famous porphyria case Petry.†

A knowledge of the I : III ratio normally excreted is now most imperative for the interpretation of the evidence accumulating from pathological conditions. Subjects should be studied separately, as pooling of extracts may lead to a result of doubtful significance.‡ We have found a I : III ratio of 4·3 : 1 in one normal urine and both faeces and urine of others are under examination.

* Maechling, E. H. [1940–1]. *J. Lab. Clin. Med.* **26**, 1676.

† Mertens, E. [1939]. *Hoppe-Seyl. Z.* **258**, Suppl. 1.

‡ E.g. Grotepass, W. [1938], *Hoppe-Seyl. Z.* **253**, 276, who used 10,000 l. of pooled hospital urine and obtained an equivocal result.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **228th Meeting** *of the Biochemical Society was held in the Department of Medical Chemistry, the University, Edinburgh, on Saturday, 5 June* 1943, *at* 2.30 *p.m., when the following papers were read:*

Heated Cholesterol as a Potential Carcinogen.

By A. H. M. KIRBY

The high proportion of human cancer deaths due to stomach cancer and the extreme rarity of animal stomach cancer suggest a carcinogen in human food. Roffo* claimed adenocarcinoma in rats fed fats or cholesterol heated ½-hour to 350° C. Beck & Peacock† were unable to confirm for heated fats but found papillomatosis of forestomach due to induced avitaminosis-A.

Groups of Wistar rats (on basal diet of rat-cake,‡ water *ad lib.*, milk and greenstuff) were fed:

(1) Unheated cholesterol: 20 mg. each/day (controls).

(2) Cholesterol heated to 270–300° C. for ½-hour (unchanged sterol removed): 20 mg. each/day.

(3) Residue from (2) after cholesterone and dicholesteryl ether removed: 20 mg. each/day.

(4) $\Delta_{3,5}$ Cholestadiene (claimed carcinogenic by Waterman§): 25 mg. each/day.

Only hyperkeratosis and 1 papilloma seen in two years. No tumour seen in other rats fed heated cholesterol esters or cholesterol heated to 430° C. (fifteen months).

Solutions of above products in olive or arachis oil injected subcutaneously into mice by Dr S. Beck; only two fibrosarcomata seen in total of 90 mice (Steiner, Steele & Koch¶).

Roffo's claim that heated cholesterol contains a polycyclic hydrocarbon very similar to 3:4-benzpyrene not confirmed. Substance with strong blue fluorescence in U.-V. beam present, but absorption maxima lie at 2330; 2440; 2560 and 2615 Å.

* Roffo, A. H. [1939]. *Bull. Ass. franç. Cancer*, **28**, 556; *Z. Krebsforsch.* **49**, 341.
† Beck, S. & Peacock, P. R. [1941]. *Brit. Med. J.* (ii), 81.
‡ Thomson, W. [1936]. *J. Hyg., Camb.*, **36**, 24.
§ Waterman, N. [1939]. *Acta Union Int. contre Cancer*, **4**, 764.
¶ Steiner, P. E., Steele, R. & Koch, F. C. [1943]. *Cancer Res.* **3**, 100.

The State of Vitamin A and Carotene in Human Blood Plasma.

By DZIAOSZYNSKI, E. M. MYSTKOWSKI and C. P. STEWART

The Ribonucleic Acid Content of Mammalian Tissues.

By J. N. DAVIDSON and C. WAYMOUTH

It is now recognized that ribonucleic acids (yeast nucleic acid, phytonucleic acid) are not found exclusively in yeast cells and plant tissues but occur also in animal tissues, e.g. pancreas, along with desoxyribonucleic acid (thymonucleic acid, animal nucleic acid).

Using chiefly material from the adult and embryo sheep we have demonstrated the presence of ribonucleic acids in all tissues so far examined. The nucleic acids were extracted from the tissues by means of 10 % sodium chloride and were precipitated as lanthanum salts. Ribose and desoxyribose were colorimetrically estimated in the precipitate with the aid of the Spekker Absorbtiometer using calibration curves drawn

up in terms of ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP). The presence of ribonucleic acid was confirmed enzymatically with the aid of crystalline ribonuclease* which attacks specifically ribonucleic acids.

Although in most tissues the amount of total nucleic acid was much higher in the embryo than in the adult, the ratio of RNAP to DNAP was either of the same order in the embryo as in the adult or was somewhat higher in the latter. The ratio was high in such tissues as brain, heart, and liver and low in spleen and thymus.

Some of the results obtained support the view that ribonucleic acid preponderates in the cytoplasm and desoxyribonucleic acid in the nuclei.

* Kunitz, M. [1940]. *J. Gen. Physiol.* **24**, 15.

The Response of Protein Metabolism to Injury. By H. N. Munro and D. P. Cuthbertson

Both in the human being and in the rat, injury such as the fracture of a long bone causes a marked rise in the excretion of N, P, K and creatine which is maximal about the third day after injury (Cuthbertson, M'Girr & Robertson*). As this excessive excretion of N could not be accounted for by loss of tissue substance locally at the site of fracture, it was concluded that a generalized tissue breakdown took place to meet the emergency.

Pursuing the origin of this N further, we have studied the response to fracture of rats when they are fed on a protein-free but otherwise qualitatively adequate diet slightly deficient in energy value. Two sets of 4 rats each were fed on a protein-free diet until the N excretion fell to a steady level; fracture of the left femur was then carried out and their urinary N output followed on the same dietary regime for a further 8 days. No rise in N output was found in either group. An identical operative procedure on animals fed at two different levels of protein intake gave metabolic responses similar to those reported in previous publications.

We are therefore persuaded that the excessive output of N which follows injury arises from storage-protein and not from essential tissue substance.

* Cuthbertson, D. P., M'Girr, J. L. & Robertson, J. S. M. [1939]. *Quart. J. Med.* **29**, 13.

Investigations on the Activity of Histaminase in Normal and Toxaemic Pregnancy. By R. Kapeller-Adler

An Inexpensive Balanced Photoelectric Colorimeter for Mains Operation. By G. H. Bell and A. R. Smellie (introduced by G. M. Wishart)

The photoelectric haemoglobinometer shown by Bell & Guthmann* to the Society last year has proved to be very satisfactory; the lamp cannot, however, be supplied from the mains. In the present form two photocells and two light filters are required; the photocells are placed equidistant from a single light and are connected in parallel opposition directly to the galvanometer. With water in the glass cells in front of the photocells the area of illumination of the reference photocell is altered by a shutter until the galvanometer is brought to zero. The circuit is now balanced for quite considerable light variations; the lamp can be supplied from A.C. or D.C. mains. The rest of the procedure, including the calibration of the E scale, is as previously described.

* Bell, G. H. & Guthmann, E. [1942]. *Biochem. J.* **36**, *Proc.* 6 June 1942, iii.

The Vitamin P Potency of Foodstuffs. By H. SCARBOROUGH

By determinations of alterations in capillary resistance in man, an attempt has been made to assess the vitamin P potency of certain fruits and vegetables. 'Good' sources of the vitamin include grapes and grape juice, both dark and light varieties, lemons and orange juice, pulp and peel, plums, especially the darker coloured varieties, prunes, 'citrin' prepared according to the method of Szent-Györgyi (supplied by Roche Products Ltd.), a water-soluble concentrate prepared from blackcurrants by Dr A. Pollard, Long Ashton Research Station (W.S.P. 4, Bacharach & Coates*) and preparations of rose hips (supplied by Dr F. Wokes, Ovaltine Research Laboratories). 'Moderately good' sources include grape-fruit juice and pulp, dried apricots, cherries, blackberries and rose-hip purée (prepared by M. Andross†). 'Poor' sources include most green vegetables, tomatoes, potatoes and a blackcurrant purée (supplied by M. Oliver, Chivers Ltd.).

On the basis of the data obtained by Bacharach & Coates with guinea-pigs, 'good' sources contain more than 100 'provisional units' per 100 g.; 'moderate' sources contain approximately 100 units per 100 g.; 'poor' sources contain under 100 units per 100 g.

* Bacharach, A. L. & Coates, M. E. [1942]. *Analyst*, **67**, 313.
† Andross, M. [1941]. *Analyst*, **66**, 358.

The **229th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University of Sheffield, on Saturday,* 10 *July* 1943, *at* 2.30 *p.m., when the following papers were read:*

Pantoyltaurine-resistance in Bacteria; the Experimental Derivation of Drug-resistant Strains in the Absence of any Inhibitor. By H. MCILWAIN

Different strains of *Corynebacterium diphtheriae* showed varying degrees of resistance to pantoyltaurine, which was partly correlated with their ability to grow with β-alanine in place of pantothenate; sensitive strains required added pantothenate. The correlation was first found in naturally occurring strains but extended to those which were made resistant to the drug by growth in its presence. Typical pantoyltaurine-fast strains synthesized pantothenate, and were also prepared by training strains to grow without added pantothenate. Pantothenate synthesis is thus a major factor in their pantoyltaurine resistance.

Natural strains of group A haemolytic streptococci were uniformly sensitive to pantoyltaurine, but sensitive organisms of this and other groups were made resistant to the drug by suitably arranged growth in its presence. Resistance in these and in some other streptococcal strains was not by pantothenate synthesis. Some organisms insensitive to pantoyltaurine alone were made sensitive to it by the presence of salicylate in sub-inhibitory concentrations. Such synergism was specific to salicylate. The joint inhibition was antagonized by pantothenate, which also antagonized inhibitions due to limiting concentrations of salicylate alone. These organisms, also, appear to owe their resistance to a modification of processes which involve pantothenate.

Partition Chromatography of Free Amino-acids and Peptides. By A. H. GORDON, A. J. P. MARTIN and R. L. M. SYNGE

The data of England & Cohn* on the distribution of amino-acids between BuOH-H_2O phases suggest that this system, originally employed for analytical purposes by Dakin, should give good separations of amino-acids when used in a partition chromatogram.

When silica gel is employed to hold the aqueous phase, poor resolutions are obtained, presumably owing to adsorption by the silica of the substances which it is desired to separate. By substitution of cellulose for silica more promising results have been obtained.

* England & Cohn [1935]. *J. Amer. chem. Soc.* **57**, 634.

An Oxidase System in the Potato Tuber. By J. G. Boswell

The Protection of Tocopherol during Saponification with Pyrogallol. By T. Moore and J. Tošić

The instability of tocopherol to alkali presents a serious difficulty in the estimation of the vitamin by the method of Emmerie & Engel.* During the usual procedure of rapid saponification of the fatty medium with hot alcoholic potash much vitamin may be lost. On the other hand, the reaction of Emmerie & Engel is not given by tocopherol acetate, which suggests that vitamin present in esterified forms in natural sources might escape estimation if saponification were to be omitted. The destruction of the vitamin presumably depends on the presence of oxygen, but Lester Smith & Bailey† found that rapid saponification in an atmosphere of inert gas gave no higher results than their ordinary procedure, in which 87 % of synthetic α-tocopherol added to wheat-germ oil was recovered.

We have found by means of recovery tests that loss may be completely prevented by the convenient procedure of adding pyrogallol before saponification. The pyrogallol is removed during the usual washing of the ether layer (with 0·5 % potash and water) before evaporating. The following results have been obtained:

Recovery of α-tocopherol during saponification in presence and absence of pyrogallol

α-Tocopherol mg. added	Without pyrogallol		With pyrogallol	
	mg. found	% recovery	mg. found	% recovery
	α-Tocopherol acetate in absence of oil			
1·78	0·66	37·4	1·76	98·8
1·78	1·04	58·5	1·77	99·4
	α-Tocopherol acetate dissolved in arachis oil			
2·06*	1·84	89·3	2·02	98·2
2·06	1·80	87·4	2·03	98·6

* Vitamin E present in the oil is made up as follows: 1·82 mg. in the form of added synthetic α-tocopherol acetate + 0·24 mg. found in the arachis oil itself by our pyrogallol method.

The much smaller loss of tocopherol when dissolved in arachis oil may be due to the presence in the fat of substances capable at least of reducing the amount of oxygen available for the destruction of the vitamin. Experience with numerous vegetable fats has shown that the pyrogallol procedure gives consistent results. Without pyrogallol substantially lower values are obtained, the extent of the deficit depending on the nature of the oil.

The value of pyrogallol for the protection of vitamin E during the digestion of tissues and foodstuffs, with alkali, and for the preservation of other unstable substances under similar circumstances is under investigation.

* Emmerie, A. & Engel, C. [1938]. *Rec. Trav. chim.* **57**, 1351.

† Lester Smith, E. & Bailey, R. [1939]. Vitamin E. A symposium, p. 21. Cambridge: Heffer.

The Effect of Glycocyamine on the Formation of Tumours induced by Acetyl-amino-fluorene. By F. BIELSCHOWSKY (introduced by H. A. KREBS)

2-Acetyl-amino-fluorene is a carcinogenic agent for the white rat.* 4 mg. of this compound (per rat and day), added to the standard diet for 20–30 weeks, produce malignant tumours in different organs. In female rats the majority of these tumours are adenocarcinomas of the breast which become evident on average after 250 days.

The addition of 20 mg. of glycocyamine (per rat and day) to the acetyl-amino-fluorene containing diet accelerates the appearance of breast tumours and at the same time increases considerably the number of liver tumours in female rats.

These results suggest that the reduction of available labile methyl-groups brought about by the conversion of glycocyamine into creatine enhances the carcinogenic action of 2-acetyl-amino-fluorene.

* Wilson, R. H., DeEds, F. & Cox, A. J. [1941]. *Cancer Res.* **1**, 595.

Determination of Isocitric Acid in Biological Material. By H. A. KREBS and L. V. EGGLESTON

The neutral solution of (−)isocitric acid is incubated with aconitase and the citric acid formed is estimated. Aconitase, unlike the related enzyme fumarase, is very labile in aqueous tissue extracts, but the enzyme is stable for several weeks in frozen minced pigeon breast muscle stored at −1°. Citric acid is determined by a modification of the volumetric method of Pucher, Vickery & Leavenworth.* For the calculation of the isocitric-acid concentration it is necessary to know the composition of the equilibrium mixture of citric, isocitric and *cis*-aconitic acids in the presence of aconitase. It was found to contain 89·5% citric, 6·2% isocitric and 4·3% aconitic acid. The higher estimate of isocitric acid of earlier authors, based on polarimetric measurements, arose from the assumption that $[\alpha]_D$ for the molybdate complex of isocitric acid was, under the test conditions, −428°. Citrate, however, increases $[\alpha]_D$ of the complex to −780°. If a is the amount of citric acid in the sample before, and b the amount after, treatment with aconitase the quantity of isocitric acid in the sample is $1{\cdot}117 \times b - a$. The occurrence of isocitric acid in various plants† is confirmed.

* Pucher, G. W., Vickery, H. B. & Leavenworth, C. S. [1924]. *Industr. Engng Chem.* (Anal. ed.), **6**, 190.
† Pucher, G. W. & Vickery, H. B. [1942]. *J. biol. Chem.* **145**, 525.

The Oxidation of Acetic Acid in Acetobacter. By J. TOŠIĆ and H. A. KREBS

Acetic acid is an end-product in *Acetobacter* when ethanol is present as a substrate, but is readily oxidized under other conditions. This observation was investigated in detail as it was thought that it might shed light on the mechanism of the biological oxidation of acetic acid. Washed suspensions of *Acetobacter turbidans* were used for most experiments. Suspensions of the organism do not absorb oxygen immediately on addition of acetate, but acquire the ability to oxidize acetate during a lag period, the duration of which increases on storage. The lag period is abolished or reduced, and the rate of oxidation of acetate is increased, by the addition of small quantities of pyruvate or some other oxidizable substances (lactate, glucose, malate, α-ketoglutarate); ethanol and propanol have no effect. In order to test whether the action of the various substrates on the oxidation of acetate is due to the synthesis of a specific catalyst during the oxidation of the substrates the effect of the B vitamins and other compounds on the lag period was tested. None of the vitamins was able to replace the oxidizable substrate. Adenyl-pyrophosphate shortened the lag period and increased the rate of acetate oxidation, but the effect was somewhat smaller than that of pyruvate.

The **230th Meeting** *of the Biochemical Society was held at St Thomas's Hospital Medical School, Manor House, Godalming, Surrey, on Saturday, 25 September* 1943, *at* 12 *noon, when the following papers were read:*

Further Observations on the *C. diphtheriae* Growth Factor of the B Complex. By F. W. Chattaway, F. C. Happold and Mary Sandford

The paper constitutes a further report on the properties of the *C. diphtheriae* growth factor present in the amyl-alcohol insoluble residues of extracts of whole liver.

The factor is relatively stable to acid, and is markedly labile to alkali, nitrous acid, ninhydrin and hydrogen peroxide. Methylation and acetylation are highly destructive and activity cannot be regained by acid hydrolysis. Adsorption occurs on norite charcoal but not on Fuller's earth. Extraction cannot be effected by diethyl ether, chloroform, petroleum ether, amyl alcohol, butyl alcohol or *p*-cresol at determined *p*H values. Precipitation of the active component does not occur with silver salts at acid and neutral reactions, and occurs only slightly with silver sulphate in the presence of baryta at *p*H values above 8·0.

Lead acetate is ineffective as a precipitating agent at *p*H values of 2·8, 6·9 and 9 to 10.

Phosphotungstic acid in 5 % sulphuric acid removes inert material from solution.

The activity of the most purified preparations is such that the addition of 5γ dried material per ml. of basal medium promotes moderate growth whereas one-hundredth of this amount evokes a detectable growth response. It is free from all traces of *L. casei* ϵ growth factor.

A Tissue Growth-Promoting Factor present in Pancreatin. By J. N. Davidson and C. Waymouth

Commercial pancreatin contains a factor which stimulates the growth in roller tubes by Willmer's* technique of fresh explants of the 9-day chick embryo heart.† When the factor is added along with embryo extract to roller tubes containing six fresh explants (embedded in fowl plasma) which have been cultivated in Tyrode solution for 48 hr., it produces in the course of a further 48 hr. a much greater rise in nucleoprotein phosphorus (N.P.P.) in the cultures than does embryo extract alone.

Composition of fluid phase in roller tubes in second 48 hr. of test	Change in N.P.P. μg. per roller tube
Embryo extract alone	+0·36
Embryo extract + crude factor	+0·82
Embryo extract + factor heated at 100° for 30 min. at *p*H 10	+0·39
Tyrode solution alone	−0·09
Purified factor (N = 50 mg./100 ml.) in Tyrode *without* embryo extract	+0·66

The factor is stable to heat in neutral or slightly acid solution, but is inactivated by heating in faintly alkaline solution. It can be purified by extraction with phenol and the purified material exerts a powerful growth-promoting action on chick heart fibroblasts even in the absence of embryo extract.

* Willmer, E. N. [1942]. *J. exp. Biol.* **19**, 11.

† Davidson, J. N. & Waymouth, C. [1943]. *Biochem. J.* **37**, 271.

Studies on the Plasma Fibrinogen and other Protein Fractions in a Case of Afibrinogenaemia. By J. L. Pinniger and F. T. G. Prunty

By adaptation of the Kark and Soutar* prothrombin technique, and from precipitin studies, evidence is presented for complete lack of fibrinogen in a girl with haemorrhagic

symptoms. The relation of the *in vitro*† and *in vivo* fibrinogen to the formation of clots, and the effects of transfusion of concentrated plasma on the protein fractions of the patient's plasma have been investigated.

* Kark & Soutar [1940]. *Lancet*, 1, 1149.
† Witts, L. J. [1942]. *J. Path. Bact.* 54, 516.

Fluorescent Derivatives of N-Methyl-Chloro-Nicotinamide. By R. A. Coulson and P. Ellinger

The supposition that F_2 might be thiochrome* proved to be incorrect.† The analogy with the elimination of methylated products after the intake of pyridine‡ and nicotinic acid§ suggested the possibility of the formation of methyl derivatives of nicotinamide which might be related to F_2.†‖ The natural non-fluorescent precursor of F_2 was crystallized from KCl eluates of urine rich in the compound by low temperature vacuum distillation, fractional precipitation of KCl and other impurities, and by adsorption on Al_2O_3 and elution with methanol. An orange aurate (m.p. 164°), a yellow mercury-potassium iodide (m.p. 136·3°) and a yellow picrate (m.p. 190·2°) were formed from the concentrate. The melting-points of the latter two were identical to the corresponding salts of synthetic N-methyl-chloro-nicotinamide†¶ and the mixed melting-points showed no depression. The aurate differed in melting-point and colour (synthetic: 170·2° yellow) (possibly two aurates as in trigonelline).**

The transformation by alkali of N-methyl-chloro-nicotinamide into two fluorescent compounds F_{2a} and F_{2b}† is nearly complete in dry isobutanol, whereas in water it is incomplete and the destruction is rapid (cf. formation of a carbinol from N-methyl-pyridium hydroxide††). The maximum yield by extraction of the alkaline aqueous phase by isobutanol is only 20 %. The predominance of the quaternary ammonium compound in water and of the carbinol† in isobutanol is evident from the spectrographs (prepared by Dr E. M. F. Roe), the shift of which resembles that obtained for the non-reduced and reduced N-methyl-iodo-nicotinamide‡‡ respectively.

* Coulson, R.A., Ellinger, P. & Platt, B. S. [1942]. *Biochem. J.* **36**, xii.
† Ellinger, P. & Coulson, R.A. [1943]. *Nature, Lond.* (in the Press).
‡ His, W. [1887]. *Arch. exp. Path. Pharmak.* **22**, 253. Cohn, R. [1893]. *Hoppe-Seyl. Z.* **18**, 16; and many others.
§ Ackermann, D. [1913]. *Z. Biol.* **59**, 18. Linneweh, W. & Reinwein, H. [1932]. *Hoppe-Zeyl. Z.* **207**, 48; **209**, 110.
‖ Najjar, V. A., Scott, D. B. M. & Holt, jr., L.E. [1943]. *Science*, **97**, 537. Huff, J. W. & Perlzweig, W. A. [1943]. *Science*, **97**, 538.
¶ Karrer, P., Schwarzenbach, G., Benz, F. & Sollmsen, V. [1936]. *Helv. Chim. Acta*, **19**, 826.
** Jahns, E. [1885]. *Ber. dtsch. chem. Ges.* **18**, 2518; [1887] **20**, 2840.
†† Hantzsch, A. & Kalb [1899]. *Ber. dtsch. chem. Ges.* **32**, 3117; Decker, E. & Kaufmann, A. [1911]. *J. prakt. Chem.* [2] **84**, 432.
‡‡ Warburg, O. & Christian, W. [1936]. *Biochem. Z.* **287**, 315.

The Excretion of Histidine in Urine. By J. R. O'Brien and P. E. Quelch

Studies in the metabolism of histidine* in pregnancy have raised problems, the solution of which would be aided by a sound method of estimation. One group of methods uses Knoop's reaction for this amino-acid which, although almost specific for histidine,† is sensitive to urinary constituents such as uric acid, phosphate and urea. To eliminate interfering substances a method based upon a base-exchange between histidine and permutite was worked out for urine. Acidified urine is allowed to percolate through a column of permutite, which is then washed with water and eluted with a saturated solution of KCl. The concentration of the purple product of Knoop's reaction on the eluate is measured photometrically.

Results obtained by this method were: Normal individuals differed greatly in the daily amounts of urinary histidine excreted—values from 0–400 mg. per day were obtained. The excretion of histidine fluctuated throughout the day and seemed related to the urinary output. Early morning specimens of urines from normals contained 0–30 mg./100 ml. In pregnancy, early morning specimens contained usually 30–100 mg./100 ml. In pregnancy complicated by toxaemia, threatened abortion or diabetes, the urinary histidine fell to values below the normal. From the present data, it would seem that estimation of urinary histidine is a useful rather than a reliable diagnostic aid in pregnancy.

* Kapeller-Adler, R. & Adler, E. [1943]. *J. Obstet. Gynaec.* **50**, 177.
† Armstrong, A. R. & Walker, E. [1932]. *Biochem. J.* **26**, 143.

The Osmotic Pressure of Foetal Haemoglobin. By E. F. McCarthy and G. Popják

The oxygen dissociation curves of foetal and maternal haemoglobin solutions with identical concentrations of hydrogen ions and salts, have been shown to differ markedly in position. In addition there seems to be a slight difference in the shape of the curves which may be represented by the calculation of n in Hill's equation $y/100 = kx^n/(1+kx^n)$, in which y represents percentage saturation, x oxygen tension and k is a constant. The value of n for foetal haemoglobin is usually slightly lower than that for maternal haemoglobin and it seemed desirable therefore to compare the degree of aggregation of foetal and maternal haemoglobin by osmotic pressure measurements.

Oxygen dissociation curves were measured on solutions of foetal and maternal haemoglobin prepared from the corresponding bloods of sheep at an early stage of pregnancy. Low values of n were obtained for foetal haemoglobin. Osmotic pressure measurements made by Adair's method showed no significant difference between foetal and maternal haemoglobin.

The Osmotic Pressure of 'Defatted' Serum. By G. Popják and E. F. McCarthy

Fresh, pooled human serum was 'defatted' by the method of McFarlane, i.e. by shaking it with ether, then freezing the mixture to below $-25°$ and thawing. The osmotic pressure of serum extracted five times in this way was compared with that of the untreated serum. The osmotic pressure measurements were made by the method of Adair. The results showed that the defatted serum exerts slightly higher osmotic pressure per gram of protein than the whole serum. However, this difference can be explained by the increase in the albumin-globulin ratio of the serum after the extraction. The conclusions are drawn that (*a*) extraction of serum by McFarlane's method removes a portion of the globulin complex, but does not alter the colloid osmotic pressure properties of the remaining proteins; and (*b*) the lipids extracted from the serum have no effect on the osmotic pressure of serum proteins.

Alloxan Diabetes (Shaw Dunn) in the Rabbit. By L. L. Ware (introduced by F. G. Young)

Vitamin C Nutrition. The Correlation of Plasma Ascorbic Acid Concentration and Urinary Saturation Tests. By F. T. G. Prunty and C. C. N. Vass

Unless the subject has recently been on an appreciably higher intake of ascorbic acid than he is now receiving, the plasma ascorbic acid concentration is a satisfactory index of the nutritional state with respect to vitamin C. Moreover, this determination is more easily carried out than a saturation test. When a 'state of saturation' is attained the plasma ascorbic acid concentration is greater than 0·8 mg./100 ml. For clinical purposes it is

suggested that a concentration of not less than 0·4 mg./100 ml. should be considered as a normal value. In general the results obtained by this determination are in agreement with those of Harris* in recent surveys.

* Harris, L. J. [1943]. *Lancet*, **1**, 515.

Succinyl Sulphathiazole and Coprosterol Formation. By O. ROSENHEIM and T. A. WEBSTER.

The bacteriostatic action of succinyl sulphathiazole on intestinal coliform bacteria was found to be concurrent with a complete inhibition of coprosterol formation. The drug was administered to rats in a diet on which about 80 % of the total sterols are excreted as coprosterol.* The conclusion that coliform bacteria are responsible for coprosterol formation was negatived by the fact that, in spite of the immediate reappearance of *B. coli* on withdrawal of the drug, the faecal sterols were found to contain only traces of coprosterol.

These and other results throw doubt on the assumed role of intestinal bacteria and suggest the possibility that the fauna rather than the flora of the intestine may be concerned in coprosterol formation. Protozoa and especially species of *trichomonas* are common parasites of the intestine of man and animals and cholesterol has been recognized as an essential growth factor for four species of *trichomonas*.†

We found that carbarsone (*p*-carbamino phenylarsonic acid), which frees the intestine from *trichomonas*, also completely inhibits coprosterol excretion. The correlation, however, appeared to be fortuitous, for rats freed from *trichomonas* by carbarsone excreted considerable amounts of coprosterol on withdrawal of the drug, but only small amounts some time after treatment with succinyl sulphathiazole, which had not freed them of the flagellates. This interference with colesterol metabolism, shared by an antibacterial and an amoebicidal drug, seems to be unconnected with their action on either bacteria or protozoa and remains so far unexplained.

* Rosenheim, O. & Webster, T. A. [1941]. *Biochem. J.* **35**, 920.
† Cailleau, R. (1937). *Ann. Inst. Pasteur*, **59**, 137, 293; (1939) *C.R. Soc. Biol., Paris*, **130**, 1089.

A Study of the Accuracy of Haemoglobin Methods. By E. J. KING, MARGARET GILCHRIST and G. E. DELORY

Three colorimetric haemoglobin methods—alkaline haematin, cyanmethaemoglobin and carboxyhaemoglobin—were tested by comparison with Fe analyses and O_2 capacity determinations. A Leitz colorimeter (Duboscq type) with mercury green light and a photoelectric colorimeter with a Chance green filter were used, with Ilford grey screens as standards. For the alkaline haematin method a haemin standard was also used. Good results were obtained on each colorimeter; photoelectric readings entail less labour. The variation of each specimen from the average colorimetric relation to iron or oxygen was calculated, and these results expressed as standard deviations.

Twelve haemoglobin preparations from horse and human bloods were found to give colours in NaOH agreeing with their iron content, when compared with standard haemin solutions.

On 20 normal bloods the standard deviations from Fe analyses and O_2 capacities were: alkaline haematin, 2·33 and 2·26 %; cyanmethaemoglobin, 1·25 and 2·18 %; carboxyhaemoglobin, 1·70 and 2·49 %; Haldane dilution tube, 3·02 %. On 20 specimens of washed laked cells from miscellaneous hospital bloods the standard deviations were: alkaline haematin, 3·30 and 2·56 %; cyanmethaemoglobin, 2·63 and 2·11 %; carboxyhaemoglobin, 4·14 and 3·32 %.

The cyanmethaemoglobin method is the most accurate; the other two give accuracy satisfactory for most purposes. The alkaline haematin method is preferred for routine work.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **231st Meeting** *of the Society was held in the Department of Organic Chemistry, The Imperial College of Science and Technology, South Kensington, London, S.W.* 7, *on Saturday,* 13 *November* 1943, *at* 11 *a.m.*

The Meeting was devoted to a discussion on 'The Tetrapyrrolic Pigments', and the following communications were made:

Properties and Function of Natural Tetrapyrrolic Compounds. By D. KEILIN

Tetrapyrrolic compounds are very widely distributed in nature, forming the active or prosthetic groups of several biologically important substances such as chlorophyll, haemoglobin, cytochrome, catalase and peroxidase. They play a fundamental role in photosynthesis, in transport of molecular oxygen and in catalysis of intracellular oxidation reactions.

The best known substance containing a tetrapyrrolic nucleus or a prophyrin is haemoglobin which is an iron-porphyrin-protein compound. By analytic and synthetic methods it has definitely been established that porphyrins are composed of four pyrrol nuclei united by four CH groups and arranged into a flat closed-ring system. In haematin the iron atom occupies the centre of this ring and of the six covalencies of Fe, four lie in one plane and connect it with nitrogens of pyrrol nuclei, while of the two others, one lies above and one below the plane of the molecule, uniting the Fe atom to nitrogenous substances such as proteins. The simplest tetrapyrrolic compound is porphin, and all the natural and artificial porphyrins can be theoretically derived from it by substitution of the H atoms of its pyrrol nuclei by methyl, ethyl, vinyl, formyl, acetic and propionic ids groups.

The porphyrins are strong and highly fluorescent pigments with sharp absorption ands. They have strong light sensibilizing properties, being therefore very toxic to organisms exposed to light. They readily combine with a great variety of metals such as Fe, Cu, Zn, Mg, Co, Mn, forming well-defined metalloporphyrins. Of these compounds only that with Fe, namely, haematin, is capable of combining with nitrogenous substances such as proteins to form either carriers of molecular oxygen (haemoglobins) or intracellular oxidation catalysts (components of cytochrome).

All the biologically important substances containing tetrapyrrolic compounds are conjugated proteins. The protein must not, however, be regarded as a mere colloidal carrier of the prosthetic group but as an integral portion of the chromoprotein molecule determining not only the magnitude but also the nature of the reaction it promotes. Thus the one prosthetic group protohaematin, combined with four different proteins, may give rise to four different substances, e.g. methaemoglobin, catalase, peroxidase, or a component of cytochrome. These highly specific proteins may therefore become one of the limiting factors in the distribution of these biologically active substances. This may explain the haphazard distribution of haemoglobin in invertebrates when one species may contain this pigment while nearly allied forms are devoid of it, or the appearance of a chromoprotein similar to haemoglobin in *Paramecium* and the nodules of leguminous plants.

The fact that tetrapyrrolic compounds have well-defined absorption spectra makes it possible to follow spectroscopically many reactions in which they are involved. In the case of cytochrome, which forms the main link between the intracellular respiratory systems and molecular oxygen, such observations can be carried out upon living cells.

It enables us to follow spectroscopically not only the respiratory activity of living cells but even to localize and determine the mechanism of action of different respiratory inhibitors. It also makes possible the observation of compound formation between a purified enzyme and its substrate.

As to the origin of the tetrapyrrolic compounds, all we can say is that cells of practically every aerobic organism are capable of synthesizing them. Only a few aerobic microorganims living normally in the presence of haemoglobin have lost this property. Their respiration and growth *in vitro* depends, however, on the addition of small amounts of protohaematin or protoporphyrin, which they can use for synthesis of their intracellular haematin catalysts. On the other hand, the anaerobic organisms are completely devoid of tetrapyrrolic compounds and of the property of synthesizing them.

The natural degradation products of haemoglobin are the bile pigments which have the structure of an open tetrapyrrolic chain with one of the porphin CH group missing. It was shown recently that the porphyrin ring of haemoglobin, while still combined with Fe and globin, may undergo an oxidative opening leading to the formation of cholegloblin. This is followed by the removal of an Fe-protein compound (haemosiderin) and the liberation of biliverdin which undergoes reduction to bilirubin. In this way the organism avoids the liberation of highly toxic porphyrins. The easy degradation of porphyrins within the living organism contrasts with their great stability outside the organism when they are protected from light and oxygen. Under such conditions porphyrins and metallo-porphyrins were found preserved for millions of years in crude mineral oils, bituminous oils, coproliths and asphalt.

Varieties of Haemoglobin. By H. Munro Fox

Chlorocruorin, a unique chemical relative of haemoglobin, which is found in the blood of certain marine annelid worms, has a haem and porphyrin differing from those of haemoglobin. The numerous varieties of haemoglobin itself, including myoglobin in muscles, all have the same haem and porphyrin; they differ from one another in their proteins. The blood pigment of vertebrate animals is distinguished from the haemoglobin (erythrocruorin) found in the blood of certain invertebrates by its molecular weight, its isoelectric point and its amino-acid composition; in these particulars chlorocruorin resembles erythrocruorin. The haemoglobins of various kinds of animals differ from one another in crystal form, spectra, oxygen affinity, resistance to alkali, sulphur content, antigenic properties, etc. There are also differences between the haemoglobins of individuals; these are among the few known individual chemical distinctions. More than one haemoglobin has been found to be present in a single individual, either successively during growth, or simultaneously.

The oxygen affinities of the different haemoglobins suit their mode of action. The haemoglobins in foetal blood, in muscle, in the blood of carp and of invertebrates living in oxygen-deficient surroundings have a high oxygen affinity; they take up oxygen even when little is available. The haemoglobins in the blood of active animals, such as trout and birds, have a low oxygen affinity; plenty of oxygen is available from outside and it is given up by the haemoglobin to the tissues at a relatively high pressure. The oxygen affinities of the blood of small invertebrates is at present being studied with an apparatus requiring only a drop of whole blood.

The actual function of haemoglobin in lower animals is not usually obvious, for invertebrates can be gassed with carbon monoxide without succumbing. The best test of its function is to eliminate haemoglobin by means of carbon monoxide and then to measure the oxygen consumption of the animal. This has been done satisfactorily only

with four invertebrates; in three of them the respiratory blood pigment functions as an oxygen carrier at atmospheric pressure of air and at pressures below this, in one it only functions at low oxygen pressures. Care must be taken in these experiments to use insufficient carbon monoxide to poison the cytochrome oxidase.

Haemoglobin is continuously produced and destroyed both in vertebrate animals and in annelid worms. Why is this so? And what keeps the haemoglobin level constant? An excess of haemoglobin is formed in response to environmental oxygen deficiency in mountaineers, in fishes and in *Daphnia*. What is the internal chemical stimulus for this synthesis?

Tetrapyrrolic Compounds in Plants. By R. Hill

With the photosynthetic activity of plants are associated the five fluorescent compounds: chlorophyll *a* and *b*, Mg-phaeoporphyrin, phycoerythrin and phycocyan. In the algae all five pigments occur; chlorophyll *a* is always present but accompanied by another fluorescent pigment often peculiar to a classified group. Phycoerthyrin and phycocyan occur together and were shown by R. Lemberg to belong to the bile pigment series. In the higher plants chlorophyll *a* is always found with chlorophyll *b*; the same association occurs in many algae. The protochlorophyll which sometimes appears in higher plants was shown to be a Mg-phaeoporphyrin by H. Fischer. Direct observations on the presence or absence of chlorophyll *b* in living cells of different algae have been made by D. W. P. Greenham, R. H. Richens and R. Hill; a previously known pigment of the Phaeophyceae they found to be a Mg-phaeoporphyrin.

Cytochrome discovered in the colourless tissues of green plants by D. Keilin can be said to have brought the plant more into the biochemist's ken. A possibility of removing and concentrating the cytochrome system as a whole from certain plant tissues was realized by K. Bhagvat and R. Hill. Catalytic properties of the preparation were examined and found to resemble similar preparations from mammalian muscle. Also oxidation and reduction of the cytochrome could readily be observed in living colourless tissues from a variety of plants. This showed that respiration passes significantly through cytochrome as in the other aerobic organisms originally examined by Keilin.

Finally, if the green plant cell is originally derived from an aerobic cell containing haematin compounds, substances intermediate between haematin and chlorophyll should exist. R. Scarisbrick and R. Hill have examined haem-protein compounds which could be obtained from green plants. In the water soluble extract of leaves from a variety of plants they found cytochrome *c* and another component having α band at 5600 A. This new haemochromogen seems to be characteristic of plant cells but is not confined to the green parts exclusively. It is autoxidizable but not rapidly enough for catalytic properties. It has sharply defined bands and does not combine with CO. In a 50 % alcoholic extract of leaves, however, another haemochromogen was found. This was designated cytochrome *f*. It has different properties from any previously known haemochromogen. The spectrum is very sharp, resembling cytochrome *c* when the latter is cooled in liquid air. Both α and β bands are complex, showing seven maxima in all: α 5550, 5510, 5450 A.; β 5330, 5250, 5140, 5080 A. The substance shows almost complete non-autoxidizability and the redox potential is positive to cytochrome *c*. It seems to be characteristic of green tissue and could be obtained in 10 times the amount of the haemochromogen 5600 A. from a given source, the ratio chlorophyll to cytochrome *f* being about 150:1. This may be regarded as a pigment more characteristic of photosynthetic cells than cytochrome proper and may represent one of the substances which determine the failure of chlorophyll production in the absence of iron.

The Constitution of Catalase. By E. F. HARTREE

In 1930 Zeile and Hellström prepared a fairly pure and high concentrated preparation of catalase which yielded crystalline protohaemin on acid hydrolysis. These authors concluded that catalase consisted of protohaematin combined with a specific protein.

No investigations of the protein have as yet been carried out but in 1935 Stern showed that treatment of catalase with acid acetone yielded a mixture of haematin and biliverdin. Lemberg showed that the biliverdin was derived from an iron-bile pigment group in the catalase. The possibility that the bile pigment was present as an impurity in the catalase preparation appeared to be ruled out when crystalline horse- and ox-liver catalases were split with acetone in the same way to yield a mixture of haematin aud biliverdin. The ratio biliverdin:haematin was found to be approximately constant (1:3) and it was therefore assumed that each molecule of catalase contained one molecule of bile pigment to three of catalase. Refinement of the analytical methods showed, however, that there were significant variations in the 1:3 ratio and that the catalytic activities of purified preparations were proportional to the concentration of haematin iron.

The evidence now available supports the view that the bile pigment groupings in catalase are catalytically inactive and arise through contact of the enzyme with hydrogen peroxide resulting from cellular metabolism. The following points lend further support: (1) crystalline catalase from blood where peroxide formation is much less than in liver is free from bile pigment; (2) the isolation of catalase from horse liver by a method normally yielding a preparation with a 1:3 ratio can sometimes yield crystalline material with an approximately 2:2 ratio; (3) treatment of catalase preparations with hydrogen peroxide can increase the ratio bile pigment:haematin.

The Metabolism of the Blood Tetrapyrrolic Pigments. By J. R. P. O'BRIEN

The metabolic fate of pyrrole pigments is bound up with the synthesis and degradation of haemoglobin. It rests particularly upon the predominating state of the bone marrow and liver. Even in adverse conditions the body can adequately supply the constituents for porphyrin synthesis. Yet globin and iron are strongly dependent upon exogenous supplies. Haemoglobin synthesis is related to the maintenance of the plasma proteins and protein stores. The mode of assemblage of the three components of haemoglobin is still obscure. Much has to be learnt of the structure of haemoglobin although the application of physical methods in the study of haemoglobin and the metallic porphyrins is giving hopeful results. With such information, differences in the properties of corpuscular haemoglobin and purified haemoglobin may be explained and the urgent need for an exact method of determination of this pigment satisfied. Methaemoglobinaemia and 'sulphaemoglobinaemia' due to drugs points to the necessity for knowledge on the conditions maintaining corpuscular haemoglobin in an active state.

In vitro studies on Hb breakdown have outstripped investigations on bile pigments in humans where they are limited to detection and estimation. From the work of Lemberg, Barkan, Fairley and others the stepwise degradation of Hb may involve methaemalbumin, cholebilin, verdohaemochromogens before bilirubin is formed. In the intestine, the changes suffered by bilirubin may yield a complex mixture of chromogens. Both the state of the liver and of the alimentary canal influence the amount of bilirubinoid pigments in the urine and faeces. It is probable that myoglobin contributes to the faecal bilirubinoids as is suggested by the elimination of mesobilifuscin in myopathies. The formation of mesobilifuscin suggests Hb degradation may proceed as far as dipyrryl methenes such as pentdyopent. As we have found, estimation of faecal bilirubinoids does not give a quantitative measure of the rate of haemoglobin breakdown in the body.

There is as yet no real biochemical means of assessing the haematopoietic activity of the bone marrow. In this problem the excretion of the coproporphyrin isomers I and III may ultimately prove useful. As we and others have found, increased excretion of isomer I is associated with increased haemopoiesis, whereas in toxic conditions and aplastic anaemia isomer III predominates. There are difficulties. The daily output of porphyrin fluctuates. Perfect separation of the isomers is difficult so that the relative amounts of the isomers even in normal conditions has not been decided. Melting-point is not a good criterion of purity of individual isomers. Slight variations in health and in the state of the liver may be associated with overproduction of porphyrins. Since the liver plays so great a part in porphyrin and bilirubinoid metabolism, we have made an intensive study of several tests for hepatic function with the ultimate object of obtaining a closer correlation between the state of the liver and pigment metabolism in humans.

The Porphyrinopathies. By C. RIMINGTON

A study of the porphyrinopathies, diseases accompanied by abnormal production or excretion of porphyrin, is essential to a proper understanding of the metabolism of the tetrapyrrolic pigments.

The porphyrins, of which only members of the isomeric series I and III have so far been found in nature, are widely distributed in small quantities throughout the animal and vegetable kingdoms; their metal complexes in the form of haemochromogens connected with cellular respiration are probably ubiquitous. Uroporphyrins are less widely distributed than protoporphyrin or the coproporphyrins. The bones and urine of the fox, squirrel, the shells of some molluscs and the red wing feathers of the African birds, the *Turacos*, all normally contain appreciable quantities of uroporphyrin. Nevertheless, its appearance in man is associated with pathological conditions.

The porphyrinopathies have been principally studied by Garrod, Günther and Fischer, and since 1935 by Waldenström, whose classification of these diseases into (1) porphyrinuria, (2) porphyria congenita, (3) porphyria cutanea tarda, (4) porphyria acuta, is that most generally acceptable. Porphyrinuria, which denotes an increase in porphyrin excreted in the urine, he separates sharply from the remaining groups, since it may be brought about *in any individual* by certain factors, e.g. hepatic dysfunction, poisoning by lead, sulphonamides, salvarsan, etc., whereas all the other porphyrinopathies rest upon an individual and generally familial idiosyncracy.

Porphyria congenita, present from birth, is accompanied by brown discoloration of the bones and light sensitivity. Its occurrence in a herd of bovines has been studied and from breeding experiments the character appears to be transmitted as a mendelian recessive. The majority of cases are males. Determination of the porphyrin excretion shows that, although this is variable, the ratio of the quantity of coproporphyrin to uroporphyrin is approximately constant.

Porphyria acuta becomes manifest about the second or third decade of life and the tendency toward this disease is a familiar character behaving as a mendelian dominant. Relatives of affected persons may, however, excrete appreciable quantities of uroporphyrin in the urine without necessarily suffering from other symptoms of the disease. Onset is usually sudden and the typical course followed is that of severe attacks of abdominal colic with constipation, vomiting, excretion of large amounts of uroporphyrin or a dipyrrimethene precursor of this porphyrin and of a urobilinoid pigment and, finally, development of a progressive paralysis which usually proves fatal.

There are several forms of the disease in which one or other of the main symptoms may predominate. Photosensitivity is usually absent.

Three principal suggestions have been put forward concerning the origin of the porphyrins in disease. Schreus regards them as derived from the degradation of haemoglobin, Duesberg as unutilized products of a deranged synthesis, whilst Fischer considers increased prophyrin production to be an atavistic step, a return to a foetal or primitive type of pigment metabolism.

Our knowledge does not yet permit of a final pronouncement on this matter. The demarcation between normal and pathological is hard to draw and only enthusiastic and painstaking research upon the biochemistry of the tetrapyrrols will supply the solution to the many problems with which we are still confronted.